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Research Paper

In vitro ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *Lannea acida* (ANACARDIACEAE) STEM BARK EXTRACTS

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Abstract

The present study was designed to evaluate the *in vitro* antioxidant and antibacterial activities of extracts of *Lannea acida* bark, generally used as herbal medicine in Nigeria and Africa at large. Antioxidant activity was determined using the DPPH, ABTS, and Lipid peroxidation scavenging ability. Whereas, antibacterial activity was performed by disk diffusion and broth microdilution assays against five reference bacterial strains comprising two gram-positive and three gram-negative bacteria. *Lannea acida* stem bark extracts showed potent antioxidant activities on all the parameters measured with respect to their concentration, and the antibacterial activities recorded by disk diffusion method showed that all the microorganisms were sensitive to the plant extracts at different concentrations. MICs of all the extracts (crude extract, flavonoid-rich and alkaloid-rich fractions) varied from 7.33±0.19 to 25.33±0.38 mg/mL^{-1} for all bacterial strains tested. The results proved that most of the microorganisms tested were sensitive to the plant extracts. These results show that the stem bark extracts of *L. acida* contain potent natural antioxidant and antibacterial principles.

Key words: *Lannea acida*, crude extract, flavonoid-rich extract, alkaloid-rich extract, antioxidant, antibacterial.

INTRODUCTION

The significance of plant extracts as sources therapeutics has gathered a great interest in recent times. According to WHO estimations, almost 80% of the population in Africa and Asia used traditional medicine to meet their healthcare needs. This is as a result of the toxicity of chemicals, high cost of chemical drugs, removal or inadequate health

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facilities especially in remote areas, which gives limitation to a suitable care of public health problems. However, understanding the mechanism of action of bacteria and fungi becomes complicated as a result of the occurrence of resistant bacteria and fungi to many orthodox antibiotics. Many occurrences of multidrug-resistant bacteria strains are commonly reported in African countries (Belmonte *et al*., 2010). One of the current problems scientists and medical practitioners face in the fight against infectious diseases is the development of resistance to the agents used to control them. Many pathogens have simultaneously acquired resistance to multiple drugs. Drug resistance has become a growing problem in the treatment of infectious diseases caused by bacteria, fungi, parasites and viruses (Alanis, 2005).

The contribution of free radicals has been established in many infectious diseases (Aderogba *et al*., 2005). These free radicals produce oxidative stress and altered or deteriorate the overall state of the body by altering the action of the immune system. Oxidative stress related diseases are on the increase in Africa, even among the young population (Willcox *et al*., 2004; Kalache *et al*., 2002). Free radicals and oxidants play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body (Davies, 2000; Pham-Huy *et al*., 2008). These free radicals are produced from the oxidation of biomolecules in the body through cellular metabolism *in vivo* or from external sources including pollution, cigarette smoking, radiation, medication etc). When an overload of free radicals cannot gradually be eliminated, their accumulation in the body generates a phenomenon called oxidative stress, which often lead to oxidative tissue damage.

It has been recognized that plant extracts contain phenolic and flavonoid compounds that possess potent antioxidant and antibacterial activities. (Da-Silva *et al*., 2006; Majhenic *et al*., 2007; Pereira *et al*., 2007).

Lannea acida belongs to the family *Anacardiaceae* commonly called, awere kogun in Akoko area of Ondo state, akogun in Ondo town. It is widely used as herbal medicine in West Africa. *Lannea acida* is one of the most widely distributed species of the *Lannea* family found in the hot and dry savannahs of sub-Saharan Africa. It has a rich history of ethnobotanical and ethno pharmacological usage in the treatment of a wide range of illnesses including malaria, rheumatism, dysentery and hemorrhoids. Extract of *L. acida*

bark is traditionally used in Nigeria as anti-abortifacient, vermifuge and to treat anal hemorrhoids, diarrhoea, dysentery, malnutrition, and debility while the leaf is used to treat rheumatism. Information provided by the traditional healer in Akoko area of Ondo state revealed that the bark aqueous or alcoholic extract is used in treatment of many infectious diseases. Even though *Lannea acida* demonstrated biological activity that validated its medicinal roles, no phyto-chemical study was performed to characterize the chemical constituents responsible for the observed activity. With this view, the present study was carried out to evaluate the antioxidant and antibacterial activities of the extracts of *Lannea acida* in order to provide scientific evidence for its continuous usage in ethno therapeutic management of infectious diseases.

MATERIALS AND METHODS

Experimental Plant Material

The stem bark of *Lannea acida* was collected from Ugbe Akoko town from a location ($7^0$15'42.9''N $5^0$15'01.9''E) of Ondo state and was authenticated at the Department of Plant Science and Biotechnology, Adekunle Ajasin University, and a sample specimen deposited at the herbarium for future reference. The samples were allowed to dry at room temperature. They were pulverized in mechanized laboratory grinder (Manesty, England) to a fine powder and weighed 1.6 kg. The powdered sample was then soaked in 5.5 L of absolute methanol. The mixture was thoroughly mixed and filtered after 72 hours using a Buchner vacuum filter. The supernatant was evaporated to dryness with a rotary evaporator. The percentage yield of the extract was determined according to the method described by Banso and Adeyemo (2006).

$$\text{Percentage yield} = \frac{\text{Weight of extract}}{\text{Weight of ground plant material}} \text{ X } 100$$

Extraction of flavonoid- rich fraction

A portion of the methanolic extract was dissolved in 100 ml (1:4) of 1% H_2SO_4 in a small flask and was hydrolyzed by heating on a water bath until the mixture was half of its volume. The mixture was then placed on ice for 15 minutes, to allow flavonoids

precipitated. The cooled solution was filtered. The filtrate (flavonoids aglycone mixture) was dissolved in 50 ml of warm 95% ethanol (50^{o}c), the resulting solution was again filtered and the filtrate was concentrated to dryness using rotary evaporator (Manuwa et al., 2015).

Extraction of Alkaloid- rich fraction

The fraction was prepared by weighed 20 g of the methanolic extract into a beaker containing 300 ml of warm distilled water (37^{o}C). The crude extract was allowed to dissolve before transferring it into a separatory funnel. Few drops of conc. H_2SO_4 were then added to make the solution acidic (the solution was tested with litmus paper). After this, it was decanted, and the residue was dissolved in water to test for acidity. The solution was decanted again and this step was repeated several times to concentrate the alkaloids extracted. Ammonia was then added until alkalinity level was achieved. The solution was tested with litmus paper, and then addition of chloroform until the complete extraction of alkaloids was obtained. After each extraction with chloroform, the test for alkaloids was carried out, in line with the method of Manuwa et al. (2015).

Phytochemical test

The methanolic extract was screened for the presence of some secondary metabolites such as saponins, tannins, alkaloids, terpenoids, steroid, quinones, flavonoids and cardiac glycosides as described by Sofowora (1993).

***In vitro* Antioxidant Assay**

DPPH Radical Scavenging Activity

The DPPH free radical scavenging assay measures the ability of an antioxidant to reduce the 2.2 diphenyl-1-1ppicrylhydrazyl radical (DPPH). DPPH is a free radical that is stable at room temperature which upon reduction by an antioxidant results in loss of absorbance at 515-517nm. The degree of loss of absorbance indicates the scavenging efficiency of the added antioxidant.

ABTS Radical Scavenging Activity

This spectrophotometric technique measures the relative ability of hydrogen-donating oxidants to scavenge the 2.2-azobis (3-ethalbenzothaizoline -6- sulfonic acid) radical cation chromogen (ABTS) in comparison with the antioxidant potency of standard amount of trolox. The reduced (ABTS) concentration by a certain amount of antioxidant is related to that of trolox, the water soluble vitamin E analogy and this gives the TEA value of the antioxidants.

Determination of Inhibition of Lipid Peroxidation

A modified Thiobarbituric Acid Reactive Substances (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenate as lipid –rich media. Egg homogenate (0.5 ml, 10 % v/v) and 0.1 ml of each extract was added to a test tube made up to 1ml with distilled water. Then, 0.05 ml FeSO4 (0.07 M) was added to induce lipid peroxidation and incubated for 30 minutes.

Thereafter, 1.5 ml of 20 % acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.8% (w/V) TBA in 1.1% sodium deodecylsulphate and 20% TCA were added and the resulting mixtures were vortexed and then heated at 95^{o}C for 60 minutes. After cooling 5.0 ml of butan-1-0l was added to each tube and centrifuged at 3000 rpm for 10 minutes.

The absorbance of the organic layer was measured at 532nm.

Antimicrobial assay.

Microorganisms and culture conditions

The bacterial strains used were Gram positive (*Staphylococcus aureus and Bacillus substilis)* and Gram negative (*Escherichia coli*, *Klebsiella pnuemoniae, Enterobacter aerogenes),* were obtained from Microbiology Department, Federal University of Technology, Akure, Nigeria and were cultured aerobically at 37 °C in nutrient agar medium. Before experimental use, cultures from solid media were sub- cultivated in liquid media, incubated for 24 hr and were used as sources of inoculums for each experiment. Antimicrobial activity was measured using agar-well diffusion method.

Antimicrobial screening of *L. acida* extracts on selected pathogens

Antibacterial activities of the plant extracts of *L. acida* were tested by disc-diffusion method. The minimal inhibition concentrations (MIC) of the extracts were determined. Five bacterial strains, *Staphylococcus aureu, and Bacillus substilis, Escherichia coli*, *Klebsiella pnuemoniae, Enterobacter aerogenes* were used in this study. The bacteria were tested for purity by culturing on nutrient agar and maintained on nutrient agar slants.

Antibacterial activity of the extracts.

Susceptibility of bacteria isolate to plant extract was determined following the BSAC diffusion method for Antimicrobial Susceptibility Testing Version 9.1 (Andrews, 2009). This test was carried out to determine the antimicrobial ability of the plant extract to inhibit the growth of the bacteria isolate.. The plate diffusion technique of Willey *et al*., (2008) was used for the antibiotic sensitivity test. Overnight cultures of the organisms were swabbed on sterile Muller Hilton solidified Agar plates using sterile swab sticks. An 8 mm sized cork borer was used to bore hole on the agar surface at equidistance, the well was filled with the diluted plant extract, a known antibiotic was used as positive control while distilled water was used as negative control. All the plates were incubated at 37 ^{0}C to 24 hours. The zones of inhibition generated by the antibiotics were measured to the nearest millimeters (mm) and interpreted as sensitive (S), Intermediate (I) and resistant (R). The zones of inhibition were measured and interpreted according to (NCCLS, 2000). The zone of inhibition were compared with that of Ciprofloxacin, Erythromycin and tetracycline

Determination of the minimum inhibitory concentration (MIC)

MICs were determined using the Mueller Hinton broth microdilution in 96 well-plates according to the National Committee for Clinical Laboratory Standards (Swenson *et al*., 2004). The broth from plant extract was only augmented with DMSO at a concentration of 1% in order to improve solubility (Coulidiati *et al*., 2009). The bacterial strains grown on nutrient agar at 37 °C for 24 hours were suspended in a saline solution (0.90%, w/v) to a turbidity of 0.5 McFarland standards (10^8 cfu mL^{-1}). The suspensions were diluted with Mueller Hinton broth to inoculate 96 well-plates containing 2-fold serial dilutions of extracts. The final inocula as determined by colony counts for the growth control

wells were approximately 10^5 cfu per well. Plates were incubated at 37°C for 24 h. MIC was recorded as a lowest extract concentration indicating no visible growth of each organism in the broth agar. All tests were performed in triplicate.

Statistical analysis: Statistical analysis of the data was performed using Graph Pad Prism Software version 7. The one - way ANOVA followed by Tukey's test was used to analyze and compare the results at a 95 % confidence level. Differences between means of treated and control group values of $p< 0.05$ were considered significant. Results were expressed as mean ± standard error of mean (SEM).

RESULTS

The percentage yields from the methanolic extracts was 18.26 % dry weight. The yield (% w/w) was calculated with respect to the dry weight of the starting material.

Table 1. Qualitative and quantitative estimation of phytoconstituents in *Lannea acida* extracts

Samples	Saponins	Flavonoid	Alkaloid	Tannins	Terpenoids	Phenols	Quinones
Qualitative Analysis	+++	+++	+++	++	+	+	+++
Quantitative Analysis	5.05 %	3.30 %	0.66 %	3.57mg/100g	0.35 mg/100g	0.18mg/100g	5.99 %

Antioxidant Activities

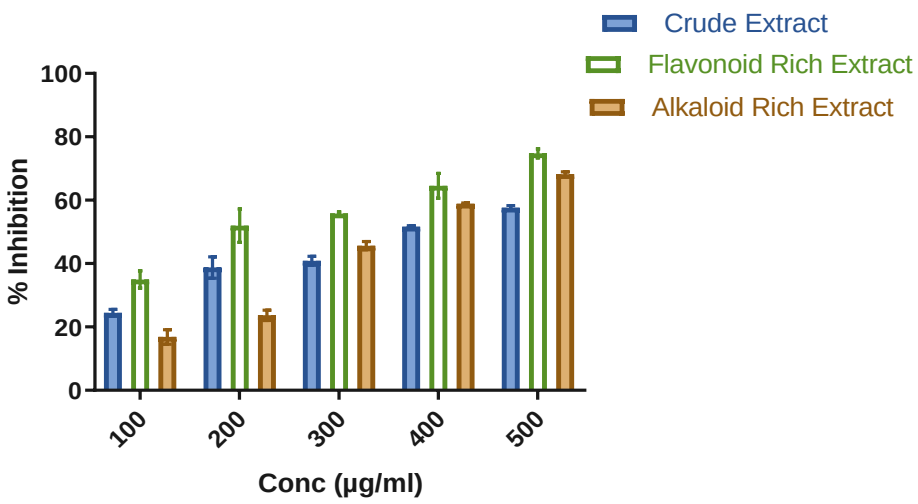

Figure 1: DPPH radical Scavenging ability of methanolic extracts of *Lannea acida*, its flavonoid and Alkaloids fractions

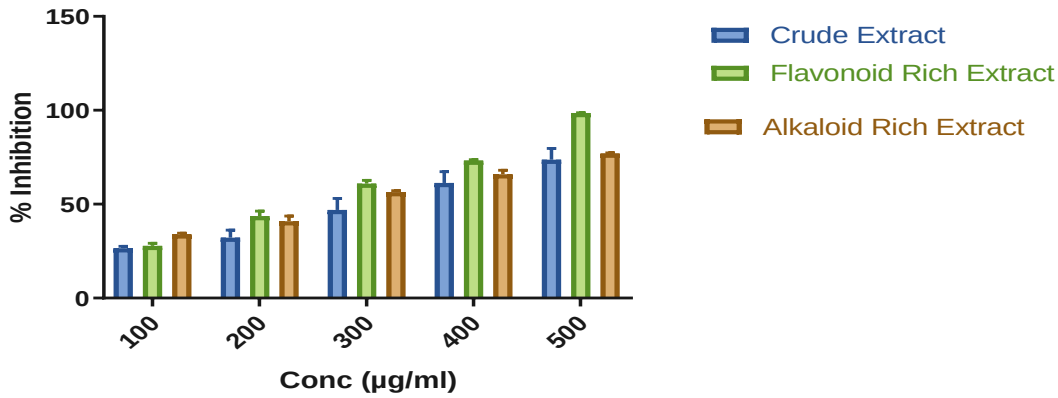

Figure 2: DPPH radical Scavenging ability of methanolic extracts of *Lannea acida*, its flavonoid and Alkaloids fractions

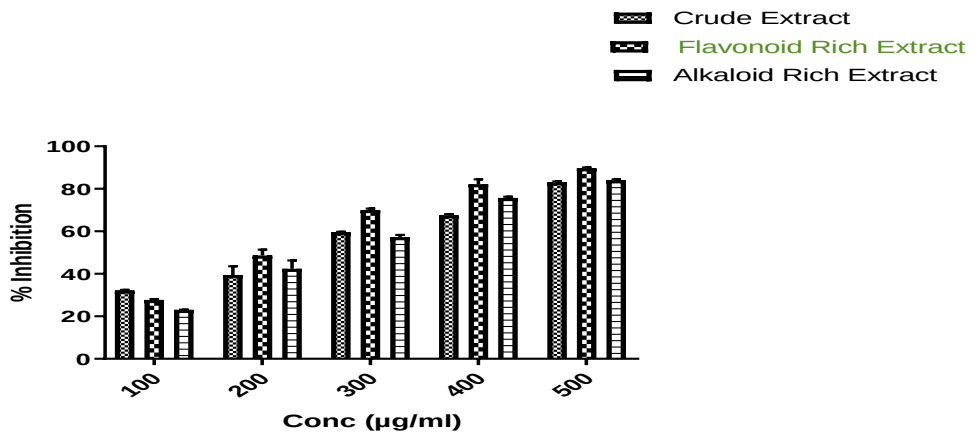

Figure 3: The inhibitory effect of methanolic extract of *Lannea acida* on Iron (II) sulphate induced lipid peroxidation in an egg yolk homogenate and its flavonoid and Alkaloids fractions.

Antibacterial Activities

Table 2: Antimicrobial effects of the methanolic extract of *L acida* plant. All values are expressed as mean inhibition zones (mm) ± SEM of three replicates. (mm)

Bacterial Strain	*L.acida* 50mg/ml	Ciprofloxacin 5µg	Erythromycin 15 µg	Tetracycline 30 µg
Gram positive				
Bacillus subtilis	17.33±0.19	34.3±0.19	26.66±0.50	20.33±0.19
Staphylococcus aureus	26.33±0.19	36.33±0.19	37.66±0.50	21.33±0.19
Gram negative				
Escherichia coil	16.66 ±0.38	37.33±0.50	36.66±0.88	30±0.33
Klebsiella pnuemoniae	23.66±0.19	32.33±0.50	29.66±1.01	23.66±0.19
Enterobacter aerogenes	13.33±0.19	12.66±0.50	9.66±0.50	38.33±0.19

Sensitive (S) ≥ 21, Intermediate (I) 20≤ 15 and resistant (R) ≤ 14

Table 3: Antimicrobial effects of Flavonoid-Rich Extract of *L acida* plant. All values are expressed as mean inhibition zones (mm) ± SEM of three replicates. (mm)

Bacterial Strain	*Flavonoid Rich* 50mg/ml	Ciprofloxacin 5µg	Erythromycin 15 µg	Tetracycline 30 µg
Gram positive				
Bacillus subtilis	18.66±0.50	31.33±0.19	26.0±0.88	21.33±0.50
Staphylococcus aureus	22.33±0.69	34.33± 0.19	33.33± 2.45	21.33±0.19
Gram negative				
Escherichia coil	23.0 ±0.33	27.66± 0.19	34.33± 1.67	28.66±0.19
Klebsiella pnuemoniae	25.33±0.50	31.66±0.19	28.66±0.50	22.66±0.50
Enterobacter aerogenes	22±0.33	10.66±0.19	8.66±0.83	27.33±0.19

Sensitive (S) ≥ 21, Intermediate (I) 20≤ 15 and resistant (R) ≤ 14

Table 4: Antimicrobial effects of the Alkaloid-Rich extract of *L acida* plant. All values are expressed as mean inhibition zones (mm) ± SEM of three replicates. (mm)

Bacterial Strain	*Alkaloid Rich* 50mg/ml	Ciprofloxacin 5µg	Erythromycin 15 µg	Tetracycline 30 µg
Gram positive				
Bacillus subtilis	10.66±0.19	31.33±0.19	26.0±0.88	21.33±0.50
Staphylococcus aureus	10.00±0.33	34.33± 0.19	33.33± 2.45	21.33±0.19
Gram negative				
Escherichia coil	8.0 ±0.33	27.66± 0.19	34.33± 1.67	28.66±0.19
Klebsiella pnuemoniae	9.33±0.19	31.66±0.19	28.66±0.50	22.66±0.50
Enterobacter aerogenes	9.33 ±0.19	10.66±0.19	8.66±0.83	27.33±0.19

Sensitive (S) ≥ 21, Intermediate (I) 20≤ 15 and resistant (R) ≤ 14

Table 5: Minimum Inhibitory concentration of methanolic extract, Flavonoid-Rich extract and Alkaloid- rich extract of *L. acida* against test microbes. (mm)

Extracts	Conc mg/ml	*B.subtilis*	*S. aureus*	*E.coli*	*K.pnuemoniae*	*E.aerogenes*
Crude Extract	50	25.33±0.19	26.33±0.19	25±0.33	20.66±0.19	26.33±0.19
	30	23.33±0.19	24.66±0.19	24.33±0.19	19.66±0.19	25.66±0.38
	20	0.00±0.00	15.33±0.19	0.00±0.00	18.66±0.19	0.00±0.00
	10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Flavonoid Fraction	50	17.33±0.19	15.33±0.19	23.33±0.19	21.66±0.19	22.66±0.38
	30	13.33±0.19	13±0.33	16.33±0.19	18.33±0.19	20±0.33

	20	10.33±0.19	10.33±0.19	12±0.33	11.33±0.19	12.33±0.50
	10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Alkaloid Fraction	50	0.00±0.00	7.33±0.19	0.00±0.00	0.00±0.00	0.00v0.00
	30	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	20	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Sensitive (S) ≥ 21, Intermediate (I) 20≤ 15 and resistant (R) ≤ 14

DISCUSSION

The result of the *in vitro* antioxidant and antibacterial activity of *Lannea acida* is present in the results. The extract produced concentration dependent increase in percentage antioxidant activity in DPPH, ABTS and lipid peroxidation assay. Also the antimicrobial activities and MIC of the extracts exhibited concentration dependent potency. The result of the preliminary phytochemical tests in table 1 indicated the presence of saponins, alkaloids, tannins, flavonoids, steroids and terpenoids. Saponins, flavonoids and alkaloids were found in high amounts, while terpenoids and tannins were moderately present. The DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The decrease in the absorbance of DPPH radical is caused by antioxidants, which react with the radical. The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared to other methods. The addition of the *Lannea acida* extract to the DPPH solution caused a rapid decrease in the absorbance at 517 nm. The degree of discoloration indicated the scavenging capacity of the extract. Figure 1 shows the concentration dependent effect of the *L. acida* extract on the DPPH radical scavenging activity. The results showed the DPPH scavenging effect of the extract with increase in concentration. The decolorization of $ABTS^{+}$ cation radical is an unambiguous way to measure the antioxidant activity of phenolic compounds. Recently, Awika *et al*. (2003) found positive correlations between the determination of phenolic antioxidant ABTS and DPPH assays. Thus, monitoring the antioxidant activity of phenolic compounds by their ability to scavenge $ABTS^{+}$ radical was demonstrated to give good prediction. The results of the ABTS are presented in Figure 2. It was observed that *L. acida* extract brought about a decrease in the concentration of the ABTS and the absorbance decreased with increase in concentration. The ABTS method is widely employed for measuring the relative radical scavenging activity of hydrogen donating

and chain breaking antioxidants in many plants extracts. It is recommended to be used for plant extracts because the long wavelength absorption maximum at 734 nm eliminates colour interference in plant extracts (Awika *et al*, 2004).

Lipid peroxidation mediated by free radicals is considered to be the major mechanism of cell membrane destruction and damage. The damage has been implicated in the pathophysiology of various human diseases such as atherosclerosis, diabetes and cancer. The initiation of peroxidation sequence in membrane or polyunsaturated fatty acids is due to the abstraction of a hydrogen atom from the double bond in the fatty acids (Wagner *et al,* 1994). Malondialdelyde (MDA) is the major product of lipid peroxidation process. Incubation of egg yolk homogenates in the presence of $FeSO_4$ causes a significant increase in lipid peroxidation. The ability of the methanolic extract of the *Lannea acida* extract to inhibit lipid peroxidation was tested using the method of Ruberto *et al.,* (2000). Figure 4 shows that the extract bring about increase in the percentage inhibition of lipid peroxidation in egg homogenate with concentration dependence. Phenolic compounds have been reported to be initiating lipid free radicals or inhibit the decomposition of hydrogen peroxide into free radicals (Maisuthisakul *et al.* 2007). Hence, the observed inhibition of lipid oxidation of *Lannea acida* extract may be due to the concentration of phenolic compounds in the plants.

The Antibacterial activity results in Table 2, 3, and 4 recorded by disk diffusion method showed that all the microorganisms were sensitive to the plant extracts and its fractions at different concentrations. MICs of the three extracts (crude extract, flavonoid-rich extract, and alkaloid rich extract) varied from 7.33±0.19 to 25.33±0.38 mg/mL^{-1} for all bacterial strains tested (Table 5). MIC values were different and suggested a selective activity of the crude extracts, and the two fractions. In order to elucidate the antibacterial effects, the zones of inhibition generated by the antibiotics were measured to the nearest millimeters (mm) and interpreted as sensitive (S),

Intermediate (I) and resistant (R). The zones of inhibition were measured and interpreted according to NCCLS (2000).

The antibacterial activity can be considerate when the diameter of inhibition zone observed is 20≤ 15 to ≥21 mm or more around the paper disk (Swenson *et al*., 2004). The results show that most of organisms tested were sensitive to the plant extracts (Table 2). The best sensitivity to the *L. acida* plant extracts was obtained on *staphylococcus aureus*, followed by *Klebsiella pnuemoniae, Bacillus subtilus*, *and Escherichia coli* while *Enterobacter aerogenes* showed resistance to *L. acida extract*. The results indicated that staphylococcus *aureus* was more sensitive to the extracts (26.33±0.19 mm) than tetracycline (21.33±0.19 mm), *Enterobacter aerogenes* was more sensitive to *L.acida* (13.33±0.19 mm) than ciprofloxacin and Erythromycin (12.66±0.50 mm; 9.66±0.50 mm).

CONCLUSION

This result obtained from this study showed potent dose-dependent *in vitro* antioxidant and antibacterial activities of the extracts. The methanolic extract and flavonoid-rich fraction of *Lannea acida* extracts appeared to be more bacteriostatic/bactericidal for almost all strains tested and demonstrated a large spectrum antibiotic with the best MICs than the alkaloid –rich fraction that indicated either a resistance to the organisms or showed no activity. However, because of the high antioxidant content of *L. acida* extract as established in the results, the stem barks extract of *Lannea acida* could be more useful in medical approach, particularly to prevent oxidative stress related diseases such as hypertension, rheumatism, cancer, prematuring aging, infectious diseases, atherosclerosis as well as a potential natural antioxidant and antibacterial agent.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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