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In Vivo Antioxidant and Immunomodulatory Activity of Flavonoids-Rich Fraction of *Lannea acida* Stem Bark

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ABSTRACT

Lannea acida is traditionally used in Nigeria as antimalarial, anti-inflammatory, antiasthmatic and antiallergic preparations. It is an ingredient of Ayurveda formulations which are used to improve immunity. The present study was performed to evaluate immunomodulatory activity of flavonoids-rich fraction of *L. acida* stem bark in rats. *In-vivo* antioxidant activity was determined using various markers of oxidative stress such as lipid peroxidation, superoxide dismutase (SOD) and catalase. The complete blood count and neutrophil adhesion were evaluated in rats randomly distributed into nine groups of five rats each. In humoral immune response model, flavonoids-rich fraction evoked a significant dose dependent increase in antibody titer values in methylprednisolone (15 mg/kg) induced immunosuppression which was sensitized with sheep red blood cells (SRBCs) on the 7th day of experiment. In cellular immune response, an increase in paw volume was recorded on the 14th day in methylprednisolone -induced immunosuppressed rats treated with SRBCs (0.1 mL of suspension containing 1×10^8 SRBCs). The fraction restored the haematological profile in methylprednisolone induced myelosuppression as well as stimulation of neutrophil adhesion percentage in the rats. The fraction showed significant protection against oxidative stress induced immunosuppression. Thus, this study significantly lends credence scientifically to claims by traditional medical practitioners that *L. acida* bark extracts have therapeutic values in managing infectious diseases through immunomodulation.

INTRODUCTION

Immunomodulation of immune system for the treatment of diseases using therapeutic plants material and their products as a possible curative measure has become an accepted therapeutic approach. Plants and minerals have been used since prehistoric times for the treatment of many disorders and diseases. It is now being accepted that immunomodulation of immune response could provide an alternative to orthodox chemotherapy for a variety of disease conditions, especially when the host's defense mechanism has to be stimulated under conditions of compromised immune responsiveness or when a selective immunosuppressant has to be induced in situation like autoimmune disorders and organ transplantation (Kaminski *et al*., 2008).

Development in clinical and experimental immunology strongly suggests that many infectious diseases and disorders arise because of stressful environmental conditions associated with suppression of immune system. It is evident that certain types of stress induce physiological changes that influence susceptibility to infection. The ability to change the immune response in animals and humans developed from a desire to confer greater protection against infectious agents through a more complete understanding of the functioning of the immune system, and of the ways in which nonspecific and specific immune mechanisms developed. Naturally occurring or synthetic compounds capable of altering those mechanisms offered further possibilities for modulating immune responses (Quinn, 1990).

Immune system is highly susceptible to oxidative damage because of the high polyunsaturated fatty acid (PUFA) content of the membranes. PUFA is a powerful potential cause of oxidative processes in the body system which often leads to membrane damage. Lipid peroxidation mediated by free radicals is considered to be the major mechanism of cell membrane destruction and cell damage. The damage has been connected to 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. The cellular and humoral components of the immune system are particularly sensitive to increased levels of reactive oxygen species which may cause gradual deterioration of the immune system. It is very important to prevent this

oxidative stress and thereby enhance the immunity of body system (Hitchon and El-Gabalawy, 2004; Ramnath *et al.*, 2009).

Lannea acida belongs to the family *Anacardiaceae* commonly called, awere kagun in Akoko area of Ondo State, Nigeria. It is widely used as herbal medicine in West Africa. *L. acida* is one of the most widely distributed of the *Lannea* species found in the hot and dry savannahs of sub-Saharan Africa. It has a rich history of ethnobotanical and ethnopharmacological usage in the treatment of a wide range of illnesses including malaria, anal haemorrhoids, diarrhea, dysentery, malnutrition and debility while the leaf is used to treat rheumatism. The stem bark is traditionally used in Nigeria as antiabortifacient and vermifuge (Olusola *et al.*, 2020).

Information provided by the traditional healer in Akoko area of Ondo State, Nigeria revealed that the stem bark aqueous or alcoholic extract is used in treatment of severe malaria. The source further revealed that when the extract is taken regularly, people appeared to be protected against malarial attacks. This points to the prophylactic effect of the plant. Thus, the present study was undertaken to evaluate the *in-vivo* antioxidant and immunomodulatory activity of flavonoid-rich fraction of *Lannea acida* stem bark.

2. MATERIALS AND METHODS

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. It was allowed to dry at room temperature and then pulverized in mechanized laboratory grinder (Manesty, England) to fine powder. The dried powder, 1.6 kg was soaked in 5.5 L of absolute methanol. The mixture was thoroughly mixed and filtered after 72 hours using a Buchner vacuum filter. The filtered supernatant was evaporated to dryness with a rotary evaporator. The percentage yield of the extract was determined according to the expression described by Banso and Adeyemo (2006).

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

A portion of the methanolic extract was then 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 placed on ice for 15 minutes, to allow flavonoids to precipitate. 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 (Ogunsina, 2020).

2.1. Drugs

Methylprednisolone sodium succinate induced immunosuppressive molecule (Pfizer Pharmaceutical, India, LOT; 839BA, MFG; 10/2015, Expiry; 09/2020) was used as a negative control, levamisole hydrochloride BP 40 mg syrup (Reals Pharmaceutical Ltd, Batch No EE48; Expiry 06/19) was used as positive control.

2.2. Experimental Animals

Wister albino rats of weight 12-26 g were used. They were divided into 9 groups of 5 rats. They were purchased from a breeding company and were acclimatized at 25 °C for two weeks, in a 12 h light/12 h dark cycle in the animal house of the standard laboratory. They have free access to commercial pellet diet and clean water *ad libitum*. Male rats were separated from their female counterparts, before and during the time of the study to avoid conception during the study time.

2.3. Antigen preparation

Fresh blood was collected from healthy sheep and mixed with sterile Alsever's solution (1:1).

The blood was then centrifuged at 3500 rpm for 5 min to enable red blood cells to settle at the bottom of the test tube. The supernatant was discarded, leaving sheep red blood cells (SRBCs) pellets.

2.4. Treatment Groups

Group I: Control group received normal saline – **Positive Control**

Group II: Control group **– Negative Control**

Group II- IX: received immunosuppressant (methylprednisolone) at doses of 15 mg/kg bd wt by subcutaneous injection on day 1.

Group III: received immunostimulant (levamisole hydrochloride 40 mg syrup) from day 1 to 14th day

Groups IV-IX: received the Extract from day 1 to 14th day at a dose range of 250 mg, 500 mg and 1000 mg.

2.5. Immunological response

2.5.1. Determination of complete blood count (CBC)

Two milliliters of fresh blood were drawn by intraventricular puncture for each of the animals in Groups (I-IX) on the 14th day into ethylenediaminetetraacetic acid (EDTA) bottle. The samples were then analyzed at the hematology laboratory, Federal Medical Centre, Owo, Ondo State, Nigeria, using an automated Beckman coulter A-T Pierce hematology analyzer (Beckman Coulter, Inc., Fullerton, CA, USA) for the complete blood cell counts.

2.5.2. Determination of neutrophil adhesion

This was carried out using the method of Thakur *et al.* (2006). On day 14 after administration of the extract, blood samples from rats in Groups (I-VI) were obtained by ventricular puncture and were analyzed for total leucocyte counts (TLC) and differential leukocyte counts (DLC). After the initial counts, the blood samples were incubated with 80 mg/mL of nylon fibers for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil were given as the neutrophil index (NI) of blood sample. Percentage of neutrophil adherence was calculated according to the formula described in (Thakur *et al.*, 2006).

$$\text{Neutrophil adhesion} = (NIu - Nit) \times 100 / NIu$$

Where, NIu, neutrophil index of untreated blood sample; Nit, neutrophil index of treated blood sample.

2.5.3. Determination of delayed-type hypersensitivity responses (DTHR)

The method described by Chong *et al.* (1998) was used for the DTHR studies. On day 7 of the study, Groups (I – VI) rats were primed by subcutaneously injecting 0.1 mL of suspension containing 1×10^8 SRBCs into the right hind footpad. The contralateral paw also received an equal volume of 0.1% phosphate buffered saline (PBS). The administration of flavonoid-rich extract was continued until the 14th day. On the 14th day, the animals were challenged by subcutaneously injecting 0.1 mL of 1×10^8 SRBCs into their left hind footpad. The extent of delayed-type hypersensitivity (DTH) response in the rats was determined by measuring the footpad thickness after 4, 8, and 24 h of challenge using Vernier calipers. The difference in the thickness of the right hind paw and the left hind paw was then used as a measure of DTH reaction and was expressed as a mean percent increment in thickness/edema.

$$\frac{\text{Left footpad challenged with antigen} - \text{Right footpad control}}{\text{Left footpad challenged with antigen}} \text{ X } 100$$

2.5.4. Determination of humoral antibody response to SRBC (hemagglutination antibody titer test)

The method described by Guatam et al. (2009) was used to determine the effect of the extract on the antibody level resulting from sensitization with SRBCs. Rats in test Groups I to III and VII to IX were immunized by injecting 0.5 mL of SRBCs intraperitoneally (i.p.) on the 7th day of the experiment. Administration of *L. acida* bark extract continued for another 7 days until day 14 and blood samples were collected by cardiac puncture. The samples were then centrifuged at 3500 rpm to get serum. Antibody titers were determined by the hemagglutination technique (Gautam *et al.*, 2009). Serial two-fold dilutions of serum were mixed with normal saline in microtiter plates of 96-well capacity and SRBCs (2.5µl of 1% SRBC prepared in normal saline) added to each of these dilution The Heamaglutination plates was then incubated at 37°C for 1 hour and then examined for hemagglutination reactions The reciprocal of the highest dilution of the test serum giving agglutination was taken as the hemagglutination antibody titer (HA units/µl).

2.6. Oxidative stress parameters

2.6.1. Lipid Peroxidation

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) as described by Vashney and Kale (1990). Under acidic condition, malondialdehyde (MDA) produced from the peroxidation of fatty acid membranes and food products react with the chromogenic reagent, 2-thiobarbituric acid to yield a pink coloured complex with maximum absorbance at 532 nm and fluorescence at 553 nm. The pink chromophore is readily extractable into organic solvents such as butanol. An aliquot of 0.4 ml of the kidney and liver homogenate was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30 % TCA was added. Then 0.5 ml of 0.75 % TBA was added and placed in a water bath for 45 minutes at 80^{o}C. This was then cooled in ice and centrifuged at 3500 rpm for 10 minutes. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. The MDA level was calculated according to the method described by Adam-vizi and Seregi (1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 10^5 $M^{-1}Cm^{-1}$.

$$\text{MDA(units/mg protein)} = \frac{\text{Absorbance xVolume of mixture}}{\text{E532nm x volume of sample xmg protein}}$$

2.6.2. Superoxide Dismutase Activity (SOD)

The liver homogenate was adequately diluted by PBS for the estimation of superoxide dismutase (SOD) activity according to the method described by Mistra and Fridovich (1972). Briefly, epinephrine undergoes auto-oxidation rapidly at pH 10.0 to produce adrenochrome, a pink colour product. The homogenates (1:20) (50 µl) of various groups were pipetted into test tubes, 1000 µl of freshly prepared 50 mM glycerine buffer and 17µl of adrenaline were added into all the tubes. The absorbance was taken at 480 nm for 2 minutes at 15 seconds interval against blank (glycerine buffer).

The SOD activity was expressed as % control

$$\frac{Absorbance\ of\ Test}{\text{Absorbance of Control}}\ \text{X}\ 100$$

2.6.3. Catalase Activity (CAT)

Catalase (CAT) activity was measured using the method described by Aebi (1974). Briefly, catalase decomposes hydrogen peroxide to form water. An aliquot of liver supernatant (10 µl) was added to a quartz cuvette and the reaction was started by the addition of freshly prepared H_2O_2 (30 mM) in phosphate buffer (50 mM, pH 7.0). The rate of H_2O_2 decomposition was measured spectrophotometrically at 750 nm for 120 seconds at 15 seconds interval. It is calculated as

$$\text{Catalase Activity} = \frac{\text{Change in absorbance} \times 4.6 \times 0.5 \text{ (total volume of assay)}}{10 \times 0.02 \times \text{total protection}} \text{ X } 100$$

4.6 = extinction coefficient

3. RESULTS

Table No. 1: Effect of varying doses of flavonoids-rich extract of *Lannea acida* on hematological parameters of winster albino rats on day 14 of the study

Parameters	Group I (N/S)	Group II (Mp 15)	Group III (Lev 40 mg) +0.1 SRBC	Group IV 250 mg +0.1 SRBC	Group V 500 mg +0.1SRBC	Group VI 1000 mg +0.1 SRBC	Group VII 250 mg +0.5 SRBC	Group VIII 500 mg +0.5 SRBC	Group IX 1000 mg +0.5 SRBC
WBC (10^3/µL)	6.76 ± 0.09	4.05 ± 0.27*	7.63 ± 0.14	4.73 ± 0.09	6.25 ± 0.15	7.53 ± 0.05	6.74 ± 0.19	6.96 ± 0.04	8.26 ± 0.05
NEUT (%)	31.66 ± 0.69	22 ± 0.33	33 ± 0.88	29 ± 0.66	32.66 ± 1.45	36.33 ± 0.38	17 ± 0.57	29.66 ± 0.50	31.66 ± 0.83
LYMP (%)	65 ± 1.45	55 ± 0.33	76 ± 0.88	69.33 ± 0.83	67 ± 1.15	78 ± 2.33	67 ± 0.57	76.66 ± 2.03	83.66 ± 1.01
MON (%)	1 ± 0.33	0.66 ± 0.19	0.00 ± 0.00	6.66 ± 0.19	0.00 ± 0.00	2 ± 0.66	1 ± 0.33	1 ± 0.3	1 ± 0.00
EO (%)	0.66 ± 0.38	1.66 ± 0.19	0.33 ± 0.192	0.66 ± 0.38	0.66± 0.19	1.66 ± 0.69	0.33 ± 0.192	1.66 ± 0.69	0.663 ± 0.192
BAS (%)	0.00 ± 0.0	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
RBC (10^3/µL)	6.89 ± 0.25	6.49 ± 0.12	5.55 ± 0.20	6.04 ± 0.02	6.61 ± 0.11	5.88 ± 0.24	6.68 ± 0.07	6.89 ± 0.14	6.71 ± 0.02
HGB (g/dL)	13.26 ± 0.15	7.03 ± 0.107	10.93 ± 0.18	9 ± 0.12	11.2 ± 0.18	11.73 ± 0.29	7.8 ± 0.31	10.33 ± 0.20	11.4 ± 0.14
HCT (%)	40.23 ± 0.40	33.36 ± 0.33	39.4 ± 0.44	37.56 ± 0.45	35.1 ± 0.16	32.83 ± 0.16	41.8 ±0.51	38.23 ± 0.40	38.8 ± 0.31
PLT (cell/ul)	552.3 ± 21.3	362.33± 6.25	659.3 ± 18.63	546 ± 11.01	524 ± 17.9	529 ± 17.77	450.6 ± 14.33	578.6 ± 6.04	599.6 ± 35.3
MCV(fl/cell)	61.1 ± 0.71	59.8 ± 1.41	61.23 ± 1.72	57.73 ± 1.25	58.5 ± 0.55	60.06 ± 0.88	63.2 ± 1.33	57.8 ± 0.38	58.13 ± 1.10
MCH(pg/cell)	17.06 ± 0.62	19.46 ± 1.66	17.7 ± 0.29	17.06 ± 0.20	16.8 ± 0.32	16.13 ± 0.41	20.7 ± 1.15	17.93 ± 0.18	18.23 ± 0.38
MCHC (g/dl)	29.96 ± 0.81	31.66 ± 0.53	29.7 ± 0.51	32.8 ± 1.22	30.5 ± 0.36	33.43 ± 0.09	30.43 ± 0.13	29.6 ± 0.06	30.93 ± 0.23

Extract given in mg/kg bwt

N/S, normal saline; MP 15, methylprednisolone 15 mg/kg bwt; Lev 40, levamisole 40 mg/kg bwt, *L. acida*

P < 0.01; comparisims were done with the means of negative control Group II Values are means ±SEM. It indicates the complete blood count in the tested rats. The test and standard groups were compared against negative control the flavonoids-rich extract of *L. acida* that was administered to the immunosuppressed rat had a stimulatory significant ($p \leq 0.05$) effect on the WBC, neutrophil, and lymphocyte counts with increasing doses.

Table No. 2: Effects of different doses of flavonoids-rich extracts on the neutrophil adhesion of Wistar albino rats.

Mean Neutrophil (±SEM) (cell/ul). Neutrophil adhesion (%)

Group	Treatment	Doses mg/kg bwt	Untreated	Treated	Neutrophil adhesion %
I	Normal Saline	N/S	1822 ± 9.95	1630.3 ± 11.93	5.357142857
II	Methylprednisolone	15	1269.3 ± 6.57	1202.6 ± 2.14	2.657178
III	Levamisole	40	2071 ± 8.64	2013.3 ± 5.09	3.287323944
IV	Flavonoids-rich extract	250	1417.6 ± 1.26	1405.6 ± 2.09	3.268627451
V	Flavonoids-rich extract	500	1671.6 ± 0.69	1641.6 ± 3.53	3.44827586
VI	Flavonoids-rich extract	1000	1976 ± 10.52	1898 ± 10.69	3.968253968

Values are presented as mean ± standard error mean (SEM). P< 0.01, comparison was done with the mean of negative control Group II. It shows the effect of different doses of flavonoids-rich extract of *L. acida* on the neutrophil adhesion of Wistar albino rats. The results connote a dose-dependent increment in the adhesion of neutrophils to the nylon fibers.

Figure 1 shows the effect of different concentrations of flavonoids-rich extract on the delayed hypersensitivity reactions in Wistar albino rats by measuring paw size. The results indicate a net increase in the thickness of the footpad in previously immunized rats.

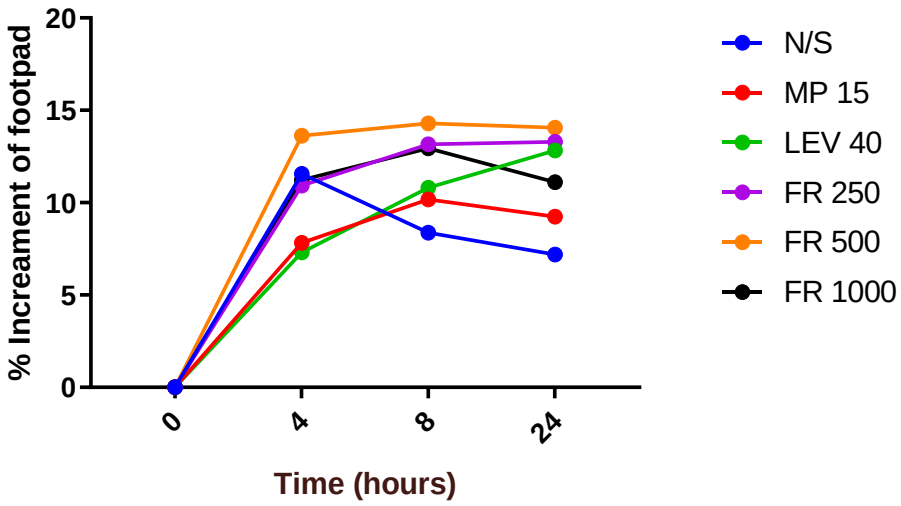

Figure No. 1: Effects of different concentrations of flavonoids-rich extract on the delayed hypersensitivity reactions in Wistar albino rats by measuring paw size. N/S, normal saline; MP 15, methylprednisolone 15 mg/kg bwt; LEV 40, levamisole 40 mg/kg bwt; FR 250, flavonoids-rich extract 250 mg/kg bwt; FR 500, flavonoids-rich extract 500 mg/kg bwt; FR 1000, flavonoids-rich extract 1000 mg/kg bwt.

Table No. 3: Effects of different doses of flavonoids-rich extracts on the of humoral antibody response to SRBCs as determined by hemagglutination antibody titres in Wistar albino rats

Group	Treatment	Doses Mg/kg bwt	Mean hemagglutination antibody titer (±SEM) HAT units/µL
I	Normal saline	N/S	5.65 ± 0.19
II	Methylprednisolone	15	4.82 ± 0.13
III	Levamisole	40	24.93 ± 0.27
VII	Flavonoids-rich extract	250	11.60 ± 0.32
VIII	Flavonoids-rich extract	500	18.60 ± 0.16
IX	Flavonoids-rich extract	1000	26.39 ± 0.16

Values are presented as mean ± standard error mean (SEM).

P< 0.01, comparisons were done with the mean of negative control Group II.

Table No. 4: Effects of flavonoids-rich extract on the oxidative stress parameters in the liver of methylprednisolone-treated rats.

Group	Treatment	LPO mg protein g/dl	SOD u/mg protein	CATALASE mmol/mg protein
I	10 mL/kg bwt N/S	3.566 ± 0.3	2.440 ± 0.09	0.169 ± 0.03
II	15 mg/kg bwt MP	5.108 ± 0.1	0.984 ± 0.06	0.044 ± 0.014
III	40 mg/kg bwt Levamisole	2.176 ± 0.3	2.183 ± 0.60	0.142 ± 0.01
IV	250 mg/kg bwt Extract 0.1 ml SRBC	3.812 ± 0.2	1.370 ± 0.21	0.050 ± 0.014
V	500 mg/kg bwt Extract 0.1 ml SRBC	1.559 ± 0.3	1.916 ± 0.01	0.377 ± 0.018
VI	1000 mg/kg bwt Extract 0.1 ml SRBC	1.141 ± 0.2	2.019 ± 0.16	0.529 ± 0.03
VII	250 mg/kg bwt Extract 0.5 ml SRBC	5.923 ± 0.8	2.039 ± 0.40	0.256 ± 0.035
VIII	500 mg/kg bwt Extract + 0.5 ml SRBC	1.782 ± 0.3	1.409 ± 0.06	0.267 ± 0.014
IX	1000 mg/kg bwt Extract + 0.5 ml SRBC	1.243 ± 0.4	1.539 ± 0.01	0.402 ± 0.019

Values are presented as mean ± standard error mean (SEM).

P< 0.01, comparisons were done with the mean of negative control Group II.

Table No. 5: Effects of flavonoids-rich extracts on oxidative stress parameters in the kidneys of methylprednisolone-treated rats.

Group	Treatment	SOD u/mg protein	CATALASE mmol/mg protein
I	10 mL/kg bwt N/S	2.917 ± 0.29[b]	0.032 ± 0.003
II	15 mg/kg bwt MP	0.338 ± 0.186	0.011 ± 0.005
III	40 mg/kg bwt Levamisole	2.132 ± 0.118	0.053 ± 0.002
IV	250 mg/kg bwt Extract 0.1 ml SRBC	1.586 ± 0.275	0.013 ± 0.022
V	500 mg/kg bwt Extract 0.1 ml SRBC	2.249 ± 0.109	0.032 ± 0.014
VI	1000 mg/kg bwt Extract 0.1 ml SRBC	2.335 ± 0.205	0.094 ± 0.042
VII	250 mg/kg bwt Extract 0.5 ml SRBC	1.499 ± 0.016	0.149 ± 0.065
VIII	500mg/kg bwt Extract + 0.5 ml SRBC	1.584 ± 0.004	0.031 ± 0.013
IX	1000mg/kg bwt Extract + 0.5 ml SRBC	1.734 ± 0.08	0.352 ± 0.153

Values are presented as mean ± standard error mean (SEM).

P< 0.01, comparisons were done with the mean of negative control Group II.

4. DISCUSSION

The immune system is susceptible to free-radical induced oxidative stress. Cellular components of immune system are very rich in polyunsaturated fatty acids which are primary target for free radicals. These free radicals are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage by causing lipid oxidation. Lipid peroxidation products formed are highly cytotoxic resulting in decrease membrane fluidity, which adversely affects immune responses (Bendich, 1993). This phenomenon may result in increased prostaglandin levels which are strong immunomodulators. The increased endogenous reactive oxygen species present during ageing and various disease states affect integral membrane function, including the cell mediated immune reaction involving phagocyte membrane NADPH oxidase that depends on the triggering of protein kinase C to produce superoxide. Depressed immunocompetence associated with ageing, various diseases and poor nutrition may result from an excess generation of reactive oxygen

species due to the downregulation of these two enzymes (Bendich, 1993; Devasagayam and Sainis, 2002).

It has been reported that antioxidant vitamins enhance immune responses but the intake needed for this action is many times greater than the currently recommended allowances (Bendich, 1993). In this circumstance, natural compounds from herbal extracts possessing antioxidant and immunomodulatory activities have tremendous potential as a prophylactic and therapeutic agent.

The endogenous antioxidant system including antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase and other molecules like bilirubin, thiols, NADPH and NADH, which catalyse free radical quenching reactions, prevents the deleterious effects of the free radicals on the immune system. Exogenous antioxidants like vitamin C, β-carotenes, vitamin E, phytonutrients function interactively and synergistically with the endogenous antioxidant system to neutralize free radicals and preserve normal function. However, impairment due to conditions like chronic inflammation, infections; exposure to allergens; and exposure to drugs or toxins such as pollution, cigarette smoke, pesticides, and insecticides may subsequently cause overutilization of endogenous antioxidants therefore rendering the immune system susceptible to free radical-induced oxidative stress leading to premature immunosenescence (Archer, 1978; Joshua *et al.*, 2015; Knight, 2000; Sies, 1997). Meanwhile, methylprednisolone was used as a tool for screening immunomodulators since it is known to impair the immune responses by inducing oxidative stress. Methylprednisolone at a dose of 15 mg/kg body weight i.p. significantly impaired the humoral, cellular and non-specific immune responses including phagocytosis, neutrophil adhesion index and haematological parameters, as compared to the control group. These results indicate that the immune system was significantly compromised. This ability of methylprednisolone to induce immunosuppression is attributed to its ability to enhance oxidative stress by generating superoxide radicals and increasing lipid peroxidation through its effect on iron release from ferritin via Fenton or Haber Weiss reaction (Bender and Meyers, 2006).

Antibody titre determination is based on the principle of agglutination reaction which is based on the ability of antibodies to cross link particles such as erythrocytes, bacterium coated with antigens to form clearly visible aggregates or agglutinates.

The observed increment in WBC counts could have been due to the presence of different nutritional elements in the extract. This agrees with previous reports which showed that the nutritional compositions of plant extracts include amino acids and fatty acids (Janeway *et al.*, 2001). Amino acids known to be very important in the synthesis of various proteins in the body including plasma proteins that play important role in the defense mechanisms of the body (Janeway *et al.*, 2001). Initial phytochemical screening carried out showed that the methanolic extract of *L. acida* contains various amino acids, vitamins, trace elements, and other elements such as iron, copper, selenium, zinc, among others (Ogunsina, 2020). These elements especially vitamins B12, B6, C, and E, and iron is essential for the synthesis of DNA and in the final maturation of the red blood cell. The amino acids found in *L. acida* are also important in the formation of globin which is essential for hemoglobin synthesis. Iron also a trace element found in *L. acida*, is one of the single most important elements in the formation of hemoglobin found in the RBC (Janeway *et al.*, 2001).

Neutrophils are part of the cell-mediated immune responses responsible for the innate immunity that contributes to the clearance of foreign bodies by recognition and migration toward the foreign body, phagocytosis, and destroying the foreign agent (Goronzy and Weyand, 2007). Results in Table 2 show a dose-dependent increment in the adhesion of neutrophils to the nylon fibers which was an indication of the boosting the neutrophil migration toward foreign bodies (Janeway *et al.*, 2001). The increased percentage of neutrophil adhesion could be attributed to the presence of the various compounds, macronutrients, and micronutrients in *L. acida* (Goronzy and Weyand, 2007).

The delay type hypersensitivity response is a type IV cell-mediated immune response according to the Coombs and Gell classification of hypersensitivity reactions. The test provides a functional *in vivo* assessment of the cell-mediated immunity. It is often used as a skin test that capitalizes on intradermal inoculation of an antigen. It is therefore used to assess the skin response following intradermal inoculation of the antigen which is dependent on antigen-specific memory T-cells and the observed results were due to the recruitment of mononuclear cells and neutrophils. Activation of the T cells leads to the release of lymphokines which cause the activation and accumulation of macrophages, increased vascular permeability, induced vasodilatation and inflammation (Bender and Meyers, 2006; Janeway et al., 2001). It also produces a boost in phagocytic activity and increases the concentration of lytic enzymes for more effective killing of microorganisms (Janeway *et al.*, 2001). Figure 1 shows a net increase

in the thickness of the footpad in previously immunized rats. This increment in footpad thickness of the Wistar albino rats that were treated with flavonoids-rich extract of *L. acida* in this study could be attributed to the ability of the extract to activate lymphocytes and their accessory cell types leading to enhancement in the production of antibodies in the immunosuppressed rats thereby increasing cell-mediated immunity. This agrees with previous reports (Banji *et al.*, 2012; Joshua *et al.*, 2015). Furthermore, *L acida* has been found to contain vitamins A, C, and K. These compounds stimulate the immune system by enhancing T-cell proliferation, increasing cytokine production and synthesis of immunoglobulins (Rodrigo *et al.*, 2008), all of which are important in the inflammatory response that was seen as an increment in the footpad thickness of the rats in this study. Amino acids also present in the *L. acida* stem bark extract are also important in the formation of immunoglobulins and major histocompatibility complexes which are essential in the mediation of the DTH reaction. Trace elements are also essential for the proliferation of the T-cells and Langerhans cells and the activity of the lytic enzymes which are important components of the DTH reaction to the antigen. This goes to show, therefore, that the flavonoids-rich extract has the potential to boost the immune system since there was a dose-dependent significant ($p \leq 0.05$) increase in the paw size in response to antigen.

A hemagglutination test was performed to determine the effect of the flavonoids-rich extract on the humoral immune response. Humoral immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation into plasma cells that secrete antibodies. Antibodies thus function as the effectors of the humoral response by binding to the antigens and neutralizing them or facilitating their elimination by cross-linking to form clusters that are then ingested by phagocytic cells (Sudha *et al.*, 2010). The results of the study (Table 3) demonstrated that flavonoid-rich extract of *L. acida* had a stimulatory effect on the humoral immune response. This was evidenced by the mean hemagglutination antibody titer to SRBCs that showed a significant ($p \leq 0.05$) dose-dependent increment for the rats treated with flavonoids-rich extract as compared to the rats that received methylprednisolone. Immunoglobulins and antigen-binding fragments are essential in the humoral immune responses that are products of amino acid chains and glycoproteins (Bender and Meyers, 2006). Copper, which is also present in *L. acida*, is essential for the functioning of the enzyme cerruloplasmin which plays part in the humoral immune response. Other compounds such as fatty acids, zinc, vitamin C, manganese, and selenium are also essential for the maturation of the B-lymphocytes in the bone marrow (Janeway *et al.*, 2001). It is apparent from this study,

therefore, that the extract contains compounds that can stimulate the production of antibodies in an immunocompromised rat.

Tables 4 and 5 indicate the effect of the flavonoids-rich extract on various oxidative stress parameters, viz. superoxide dismutase, catalase and lipid peroxidation, Lipid peroxidation is a self-propagation reaction that leads to generation of lipid radicals and lipid peroxides. During lipid oxidation, malonaldehyde (product of oxidation) can react with the free amino group of proteins, phospholipids, and nucleic acids damaging their structures and functions (Stocks and Dormandy, 1971). The negative control group showed significant increase in lipid peroxidation (5.108 ± 0.1) whereas the positive control Group showed significant reduction (2.176 ± 0.3) in lipid peroxidation induced by methylprednisolone when compared with the extract-treated Group which showed significant ($p \leq 0.05$) protection against lipid peroxidation. Superoxide dismutase is present in cell cytoplasm (copper-zinc enzyme) and in mitochondria (manganese enzyme) in order to maintain a low concentration of superoxide anion. It catalyzes the dismutation of superoxide anion into oxygen and hydrogen peroxide. There was a significant ($p \leq 0.05$) increase in the activities of SOD levels of the extract-treated Group in comparison with the Levamisole in dose-dependent activities. Catalase present in peroxisomes is a heme protein that catalyzes the detoxification of hydrogen peroxide. There was a significant ($p \leq 0.05$) increase in the activities level of catalase in both the extract and standard immunostimulants Levamisole drug (0.142 ± 0.01) groups compared to the negative control (0.044 ± 0.014).

5. CONCLUSION

It is evident from this work that flavonoids-rich fraction of stem bark of *L. acida* has *in vivo* antioxidant potential thereby confirming the immunomodulatory observations made so far. It is also very clear that the fraction has both specific and non-specific immunity modulating ability comparable to the marketed polyherbal formulations. Although the results from this work are quite promising for the use of *L. acida* fractions as antioxidant and immunomodulatory agent, further research including clinical trials is required, to make a definite conclusion. This will accelerate the integration of this medicinal plant into our health care system in the management of infectious diseases.

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Authors' contributions

All the authors designed and carried out the study. They all reviewed, read and approved the final manuscript.

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Availability of data and materials

The data sets analysed in this current study are available from the corresponding author on request.

Ethics approval and consent to participate

This study was approved by Adekunle Ajasin university Animal laboratory handling Committee.

Consent for publication

Not applicable.

Competing Interests

The authors declared that there is no competing interest.

Declaration of Conflicting Interests

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

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