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WOUND HEALING AND ANTIMICROBIAL ACTIVITIES OF THE METHANOL LEAF EXTRACT AND FRACTIONS OF *Lannea barteri* OLIV. ENGL. (ANACARDIACEAE) IN RATS

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ABSTRACT

Lannea barteri Oliv. (Anacardiaceae) gained popularity in traditional medicinal practice in the treatment of neurological disorders (madness, epilepsy and paralysis). It is mostly used to treat wounds, sores and leprosy in the western and middle belts of Nigeria. The *L. barteri* leaf extract (LBLE) and fractions were assessed for their wound healing and antimicrobial potentials. In incision wound model, 10, 20 and 40%w/w of extract and fractions were used. Gentamicin ointment (1%) and sterile soft white paraffin served as positive and negative controls respectively. The antimicrobial activity was evaluated *in vitro* using broth dilution method. The extract and fractions caused a dose-dependent significant ($p < 0.05$) increase in the wound breaking strength with 40% n-hexane fraction exhibiting the highest wound breaking strength of 650 g. All tested microorganisms were sensitive to the methanol extract. Most of the organisms were susceptible to the fractions. *E. faecalis* ATCC 29219 was resistant to the fractions. The minimal inhibitory concentrations (MICs) and minimal biocidal concentrations (MBCs) of the methanol extract and fractions against susceptible pathogens were in the range 1.67 to 100 mg/ml while the minimal fungicidal concentrations (MFCs) of the fractions against *A. fumigatus* (Af293) *and A. fumigatus* (Ku80) was 100 mg/ml. This study established that the methanol leaf extract and fractions of *L. barteri* potentiated wound healing and inhibited the growth of pathogenic microorganisms. The study thus, validated the folkloric use of *L. barteri* for the treatment of skin and wound infections and forms a strong basis for further exploration of the plant.

KEYWORDS: **Antimicrobial; Antifungal; Broth dilution; Incision wound; *Lannea barteri*; Wound healing.**

INTRODUCTION

A wound is referred to as injury or harm to the skin, tissues or organs of the body [1,2]. Damage to the body's environment from external sources leads to wound development. A change normally follows this skin breakage that can be caused by contusion, laceration, hematoma or abrasions in the structure and functions of underlying normal tissue [3]. For the tissue to regain its integrity, the damaged tissue begins the orderly process of wound healing immediately an injury occurs [4,5]. Hemostasis, inflammation, proliferation, and remodeling are some of the mechanisms involved in wound healing [6,7]. In general, wound contraction, epithelialization, and granulation tissue formation are the measurable phenomena involved in wound healing [7], with the individual contributions depending on the category of wound in question. Wound healing is a complicated process that can be hampered by a variety of factors such as infection, pollution, age, stress, oxygen, nutrition, medicine, sex hormones, obesity, diabetes,

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and venous or arterial disease, all of which contribute to chronic wounds that do not heal [8]. As a result, during wound therapy, elements that can slow down the skin's healing process should be examined.

The presence of foreign bodies and infectious pathogens in the wound prolong the inflammatory phase and thus affects healing. Bacteria hinder epithelialization, contraction, and deposition of collagen as a result of prolongation of the inflammatory phase [9]. The bacterial endotoxins trigger phagocytosis and the release of collagenase that degrades collagen and destroy the surrounding normal tissues that were already formed. Contamination of wound and tissue hypoxia greatly suppresses fibroblast proliferation which is regulated by macrophage [10]. To ensure desirable recovery, topical, oral, and systemic medications are utilized to accelerate the process of healing and decrease challenges such as severe microbial wound infection [11]. Therapeutically, drugs are used to promote wound healing process, though these drugs are usually expensive [12] and sometimes they elicit detrimental side effects to the receiver, therefore, this necessitates the search for safe and less expensive substitute or complementary substances that could enhance wound healing. Medicinal herbs are used in ethnomedicine as decoctions that are applied topically to skin wounds to help them heal faster [12]. Many plants have been found to be effective in the healing and re-establishment of wounded tissue through a variety of ways [13]. According to studies, medicinal plants are employed not only because they are inexpensive and easy to obtain, but also because they are deemed safe because they rarely produce life-threatening hypersensitivity reactions [14-18]. The efficacy of these plants in wound repair has been witnessed and passed down through generations [11].

Lannea barteri (Oliv) Engl. belongs to the family Anacardiaceae. Members of the family are abundantly found in tropical and sub-tropical regions in Africa as well as widely distributed in Nigeria. The bark is stomachic. Stomach aches, diarrhoea, swelling, paralysis, seizures, and psychosis can all be treated with a decoction of this plant [19]. Combination of this plant with some spices is useful as vermifuge in southwestern Nigeria. The bark is applied externally to cure wounds, ulcers, and leprosy [20] and a root decoction cures hernia. For decades, the stem bark of *L. barteri* has been used in northern Nigeria to cure seizures, gastritis, and juvenile convulsions, among other ailments [21]. The efficacy of this plant is generally well known among the traditional medicine practitioners. Scientific investigations on this plant have validated some of these claims such as antioxidant [19,20], acetylcholinesterase inhibitory effect, antibacterial [19], anticonvulsant [22], anticancer [23], hepatoprotective [20] and anti-inflammatory [24] actions. However, no research on the wound healing and antimicrobial properties of its leaves has been done. As a result, the wound healing and antimicrobial activities of *L. barteri* leaf extract and fractions in rats were investigated in this work.

MATERIALS AND METHODS

Materials

The chemicals, reagents, and solvents utilized were of the highest quality. They include methanol (Fluka, England), 3% Tween 80(R), Gentamicin, Ampicillin, Streptomycin and Fluconazole (Sigma-Aldrich, USA), ketamine hydrochloride, xylazine hydrochloride and chloroform.

Microbial strains and culture

Various bacterial strains that are pathogenic: *P. aeruginosa* ATCC 27853, *P. aeruginosa, S. castenllani* 51573, *E. faecalis* ATCC 29219, *S. aureus* (ATCC 27664), *Enterobacter* ATCC 13048, *S. typhymuruim* 5011, *S. aureus* 26075, *P. vulgaris* FSCC 204002 (CMCC49027), *P. mirabilis* FSCC 204001 (CMCC 49005), were employed in the research. *Staphylococcus aureus* and *E. faecalis* are Gram-positive bacteria. *Pseudomonas aeruginosa, P. mirabilis, P. vulgaris, S. typhimurium and enterobacter* are Gram-negative bacteria. *Aspergillus fumigatus* (Af293) and *Aspergillus fumigatus* (Ku80) were the fungi used. Mueller–Hinton broth/agar (Merck, USA) was used to culture the bacteria to determine the MIC and MBC while Sabouraud dextrose broth/agar medium was used for fungi.

Animals

The Department of Pharmacology and Toxicology, University of Nigeria, Nsukka, provided in-bred Swiss albino rats of either sex (170 - 220 g). The rats were housed in metal cages in the department's animal house and were given free access to food and water. They were cared for in accordance with international norms for laboratory animal use.

Plant material

Mr Alfred Ozioko (a plant taxonomist) of the International Centre for Ethnomedicine and Drug Development (Inter-CEDD), Nsukka, Nigeria, identified and authenticated fresh leaves of *Lannea barteri* obtained from the forest of Benue State,

Nigeria. The specimen voucher with No 096 was deposited in the Centre's herbarium.

Preparation, extraction and fractionation of plant material

Fresh *L. barteri* leaves were air-dried, ground into a coarse powder, weighed, and kept in an airtight container. The powder material (2000 g) was macerated for 72 h with 10 L of methanol with intermittent agitation. To obtain methanol leaf extract (MELB), the sample was filtered using Whatman no. 4 filter paper, and the filtrate concentrated using a rotary evaporator at 40°C. The weight and the percentage yield of the dried extract were determined and the extract was kept refrigerated at 4°C for further use.

To obtain the n-hexane (HFLB), ethyl acetate (EFLB), and methanol (MFLB) fractions, the MELB (100 g) was separated sequentially in a column chromatogram using n-hexane, ethyl acetate, and methanol solvents in the increasing order of polarity. The fractions were evaporated to dryness using rotary evaporator at a temperature of 40°C and their percentage yields determined. They were stored separately in amber-coloured bottles and preserved in the refrigerator.

Phytochemical screening

The extract and fractions were screened for the presence of bioactive compounds using standard methods [25,26].

Acute toxicity test (LD_{50})

Lorke's [27] method was employed to determine the acute toxicity of MELB in mice orally.

Ointment preparation

Four herbal ointments containing 10% w/w, 20% w/w, and 40% w/w of the MELB, HFLB, EFLB, and MFLB [28] were used in the study.

Incision wound model

Twenty-five albino rats were anesthetized with xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (50 mg/kg) and their dorsas were prepared for aseptic surgery [29]. Using a scalpel blade, a full paravertebral (6 cm) skin incision was made on the animals and the incision was sutured with needle (sterile traumatic needle) and thread (silk thread, size 2/0). Fourteen groups of rats, each with five animals, were treated topically as follows:

Groups A, B and C: 10, 20 and 40% w/w of MELB
Groups D, E and F: 10, 20 and 40% w/w of HFLB

Groups G, H and I: 10, 20 and 40% w/w of EFLB
Groups J, K and L: 10, 20 and 40% w/w of MFLB
Group M: 1% gentamicin ointment (positive control).
Group N: Soft white paraffin ointment base (negative control).

The rats were given daily therapy for nine days, after which the stitches were detached and the wound breaking strength was measured using the constant water flow technique on the tenth day [30].

Inoculum preparation

The bacterial and fungal suspensions were prepared by suspending 18 h grown bacterial and 24 h fungal culture in 0.89% sterile normal saline (NaCl w/v). The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard (equivalent to 1.5×10^8 cfu/ml) and 1.0 McFarland standard (equivalent to 1.5×10^8 cfu/ml) for fungal strains by placing both the McFarland standard and the inoculums tubes side by side in front of a white paper with black lines. Each of the standardized culture were also plated out and their colony/spore forming unit per ml (cfu/ml or sfu/ml) determined.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration of extracts and fractions were tested against some bacteria and fungi. Using microdilution method, extract and fractions stock solutions prepared in solvents were diluted to final concentrations of 3.125, 6.25, 12.5, 25.0, 50.0, and 100.0 mg/mL in 98 µl (two-fold serial dilution by transferring 98 µl from 196 µl of broth + each of the samples) Muller-Hinton and Sabouraud dextrose (SD) broth respectively in Eppendorf tubes. 2 µl of each standardized test microorganisms were added to each of the tubes. Similar concentrations each of the extracts and fractions were also made in tubes containing only broth as negative control, and tubes containing only broth and each of the test microorganisms were used as positive control. Antibiotics, Ampicillin, Streptomycin (bacteria) and Fluconazole (fungi) (100, 80, 40 and 20 µg/ml) were used as positive drug controls. All the tubes were incubated at 37°C and 28°C for 24 h and 48 h for bacteria and fungi, respectively. The MIC was determined as the minimum concentration of the *L. barteri* extract and fractions that inhibited microbial growth or did not show any visible growth.

Minimum biocidal concentrations (MBC) determination

A loopful of each of the culture medium tubes that did not show visible growth of the test microorganism were streaked on Mueller-Hilton and Sabouraud dextrose (SD) agar media, respectively in petri

dishes. The plates were incubated at 37°C and 28°C for 24 h and 48 h for bacteria and fungi, respectively. The growth of bacteria and fungi were observed in the culture plates. The plate streaked with the lowest concentration of *L. barteri* extract or fractions that showed no growth was used as the minimum biocidal concentration.

Statistical analysis

GraphPad Prism 5.03 was used to analyze the data, and Dunnett's post hoc test was performed. The differences between the means of the treatment and control groups were estimated at a 95 percent confidence interval and judged significant at $p<0.05$.

RESULTS

Extraction

The percentage yields of extract and fractions were 164 g (8.2%) MELB, 14.62 g (14.62%) HFLB, 9.36 g (9.36% w/w) EFLB, 20.54 g (20.54%) MFLB.

Phytochemistry

Steroids, glycosides, saponins, flavonoids, alkaloids, reducing sugar, terpenoids, proteins, tannins, carbohydrates, resins, and fat and oil all showed good results in a preliminary qualitative phytochemical examination of the extract and some of the fractions (Table 1).

Acute toxicity test

Oral administration of MELB, HFLB, EFLB, and MFLB suspended in 3% Tween 80 to mice up to 5000 mg/kg resulted in no mortality or any observable intoxication signs.

Incision wound

When compared to the control, the extract and fractions increased wound breaking strength significantly ($p<0.05$). As shown in Fig 1, the wound breaking strength rose in a dose-dependent fashion across the treatment groups, with the 40% n-hexane fraction having the maximum wound breaking strength (650.80 ± 27.80).

Minimum inhibitory concentration (MIC)

The extract and fractions inhibited the growth of both bacteria and fungi tested. The MIC values of the methanol extract against the various pathogens ranged from 1.675 to 50 mg/ml with *E. faecalis* ATCC 29219and *S. aureus* (ATCC 27664) causing the highest growth inhibitory effect at the lowest concentration of 1.675 mg/ml (Table 2). *S. castenllani* 51573, *E. faecalis* ATCC 29219, *S. aureus* (ATCC 27664) and *P. vulgaris* FSCC 204002 (CMCC49027) were resistant to the n-hexane fraction while *E. faecalis* ATCC 29219 was resistant to ethylacetate and methanol fraction (Table 2). Most of the test pathogen s were resistant to streptomycin (>100 µg/ml) unlike ampicillin which inhibited all the bacteria except *Pseudomonas aeruginosa* and *Shigella castellani* ATCC 51078. The two strains of *A. fumigatus* Af293 and Ku80 were inhibited by fluconazole (40.00 µg/ml) (Table 3).

Minimum biocidal concentration (MBC)

The minimum biocidal concentration of extract and fractions of *L. barteri* varied as shown in Table 4. The MBC values of MELB varied from 1.675 to 50 mg/ml and it had biocidal effect on all the test pathogens except the two fungi tested. The range of MBC values of the fractions HFLB, EFLB and MFLB are from 6.25 to 100, 3.13 to 100 and 3.13 to 100 mg/ml. *S. castenllani* 51573, *E. faecalis* ATCC 29219, *S. aureus* (ATCC 27664), *P. vulgaris* FSCC 204002 (CMCC49027) and *P. mirabilis* FSCC 204001 (CMCC 49005) were resistant to the n-hexane fraction while *E. faecalis* ATCC 29219 was resistant to ethylacetate and methanol fraction. Again, HFLB was least active against all the test pathogens except against the two fungi tested. Majority of the test bacteria and the two strains of *A. fumigatus* were resistant to streptomycin (unlike ampicillin) and fluconazole, respectively.

DISCUSSION

Hemostasis, inflammation, proliferation, remodeling, and maturation are all steps involved in wound healing [31]. The repair of damaged architectural integrity and functional capacity of the skin includes appropriate healing of wounds [32]. As a result, the wound healing capacity of the methanol leaf extract and fractions of *L. barteri* was investigated using an incision wound method. The amount of collagen in a healing wound tissue and the stability of the fibers determine its tensile strength [33]. Collagen is made up of amino acids and hydroxyl proline, which are employed as biochemical markers for tissue collagen. Collagen is the main component that builds and reinforces extracellular tissue [4]. Fibroblasts create a lot of collagen, which is the most important component of the extracellular wound matrix and thus is responsible for creating durability by gluing the wound borders in unison at the restored location [34]. Therefore, the observed dose dependent increase in tensile strength of wounds among the treated animals may not only be due to increased

Table 1: Phytoconstituents of extract and fractions of *L. barteri*

Constituents	ME	HF	EF	MF
Alkaloids	++	-	-	+++
Carbohydrates	++	-	-	+++
Fats and oil	++	+	-	-
Flavonoids	+++	-	-	+++
Glycosides	+++	-	-	++
Proteins	+++	-	-	+
Reducing sugars	+	-	-	++
Resins	++	+	++	+
Saponins	+	-	-	++
Steroids	++	+	-	+++
Tannins	++	-	-	++
Terpenoids	+++	+	+++	-

Key: - = absent, + = present, ++ = moderately present, +++ = abundantly present, ME = methanol extract; HF = n-hexane fraction; EF = ethylacetate fraction; MF = methanol fraction.

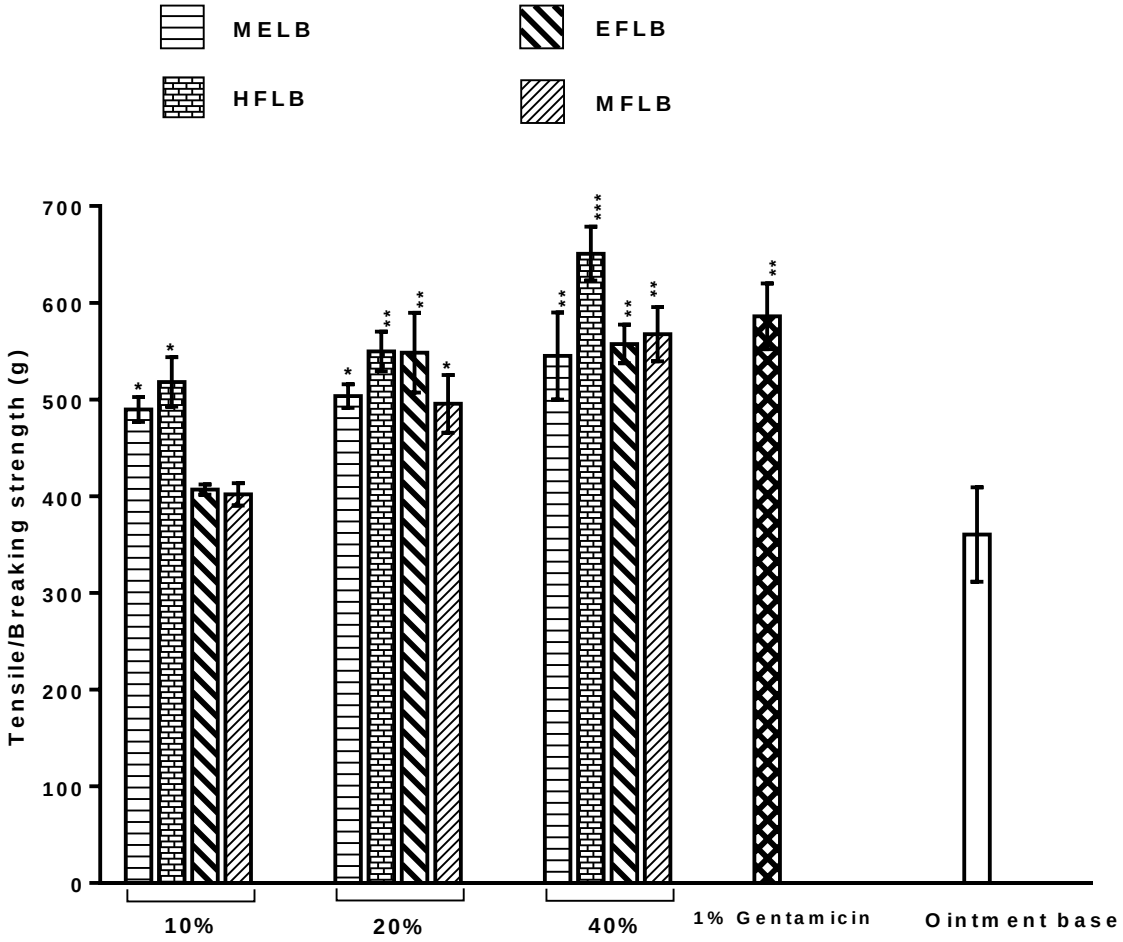

Figure 1: Effect of extract and fractions of *L. barteri* on incision wound in rats**.**
Values are mean ± SEM.; n = 5; *, **, *** Significant difference from control ($p < 0.05$, $p < 0.01$ and $p < 0.001$) respectively; MELB = methanol extract; HFLB = n-hexane fraction; EFLB = ethylacetate fraction; MFLB = methanol fraction.

Table 2: MIC analysis of extract and fractions of *L. barteri*

Microorganisms	Minimum inhibitory concentration (mg/ml)			
	MELB	HFLB	EFLB	MFLB
P. aeruginosa ATCC 27853	25.000	50.00	50.00	25.00
P. aeruginosa	12.500	50.00	50.00	25.00
S. castenllani 51573	12.500	>100.00	3.13	3.13
E. faecalis ATCC 29219	1.675	>100.00	>100.00	>100.00
S. aureus (ATCC 27664)	1.675	>100.00	3.13	3.13
Enterobacter ATCC 13048	50.000	50.00	50.00	50.00
S. typhimurium 5011	50.000	3.13	12.50	12.50
S. aureus 26075	12.500	25.00	50.00	50.00
P. vulgaris FSCC 204002 (CMCC49027)	50.000	>100.00	3.13	3.13
P. mirabilis FSCC 204001 (CMCC 49005)	50.000	100.00	25.00	12.50
Aspergillus fumigatus (Af293)	25.00	25.00	50.00	12.50
Aspergillus fumigatus (Ku80)	25.00	100.00	12.50	25.00

MELB = methanol extract; HFLB = n-hexane fraction; EFLB = ethylacetate fraction; MFLB = methanol fraction.

Table 3: MIC and MBC of the test antibiotics

S/N	Test bacteria	Antibiotics (µg/ml)					
		Ampicillin		Streptomycin		Fluconazole	
		MIC	MBC	MIC	MBC	MIC	MBC
1	*P. aeruginosa* ATCC 27853	40.0	40.0	80.0	80.0	=	=
2	*P. aeruginosa*	>100.0	>100.0	40.0	40.0	=	=
3	*S. castenllani* ATCC 51078	>100.0	>100.0	40.0	60.0	=	=
4	*E. faecalis* ATCC 29212	40.0	40.0	40.0	80.0	=	=
5	*S. aureus* ATCC 27664	40.0	40.0	>100.0	>100.0	=	=
6	*E. aerogenes*	40.0	60.0	>100.0	>100.0	=	=
7	*S. typhimurium*	40.0	60.0	>100.0	>100.0	=	=
8	*S. aureus*	40.0	40.0	>100.0	>100.0	=	=
9	*P. vulgaris* FSCC 204002 (CMCC 49027)	40.0	40.0	>100.0	>100.0	=	=
10	*P. vulgaris* FSCC 204001 (CMCC 49005)	40.0	40.0	>100.0	>100.0	=	=
11	*Aspergillus fumigatus* (Af293)	=	=	=	=	40.0	>100.0
12	*Aspergillus fumigatus* (Ku80)	=	=	=	=	40.0	>100.0

- = not tested

Table 4: MBC analysis of extract and fractions of *L. barteri*

Microorganisms	Minimum biocidal concentration (mg/ml)			
	MELB	HFLB	EFLB	MFLB
P. aeruginosa ATCC 27853	25.000	100.00	100.00	100.00
P. aeruginosa	12.500	100.00	100.00	100.00
S. castenllani 51573	12.500	>100.0	3.13	3.13
E. faecalis ATCC 29219	1.675	>100.0	>100.0	>100.0
S. aureus (ATCC 27664)	1.675	>100.0	3.13	3.13
Enterobacter ATCC 13048	50.000	100.00	100.00	100.00
S. typhimurium 5011	50.000	6.25	25.00	25.00
S. aureus 26075	25.000	100.00	100.00	100.00
P. vulgaris FSCC 204002 (CMCC49027)	50.000	>100.0	3.13	3.13
P. mirabilis FSCC 204001 (CMCC 49005)	50.000	>100.0	50.00	100.00
Aspergillus fumigatus (Af293)	>100.0	100.0	>100.0	>100.0
Aspergillus fumigatus (Ku80)	>100.0	100.0	100.0	100.0

MELB = methanol extract; HFLB = n-hexane fraction; EFLB = ethylacetate fraction; MFLB = methanol fraction.

collagen synthesis, but also due to its proper deposition and alignment [35]. Furthermore, the significantly higher wound breaking strength in the treated groups when compared to the control group implies that the *L. barteri* extract and fractions had a dose-dependent wound healing effect. Inhibitors of inflammation are expected to improve wound healing since inflammation is linked to tissue harm. Mbaoji *et al*. [24] found that the methanol leaf extract and fractions of *L. barteri* had anti-inflammatory properties.

Because an infected wound is less likely to heal, removing the infection and preventing additional infection is critical for quick and effective wound healing [36]. Infected wounds induce a large number of phagocytic cells, which emit reactive oxygen species in an effort to tackle infection; nevertheless, these substances might harm the host cells and delay recovery [37]. The minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC) values were used to assess the antibacterial activity of extract and fractions of *L. barteri* against the microorganisms studied in this investigation. Across the pathogens examined, the methanol extract and fractions had varying antibacterial and antifungal activity. The extract had the most effectiveness against sensitive species, while the hexane fraction had the least activity. *E. faecalis* ATCC 29219 was resistant to all the fractions. The hexane fraction caused the best antifungal activity. Organisms sensitive to the methanol extract and fractions could be due to interplay of adequate membrane permeability, microbial enzyme inhibition and disruption of metabolic cellular processes among other mechanisms [38]. The extract and fractions of *L. barteri* caused strong antimicrobial activity against a number of microbes including those known to infect wounds and delay its healing such as *Escherichia coli*, *Proteus spp*., *Enterobacter spp*., *Staphylococcus aureus* and *Pseudomonas aeruginosa* [39,40]. Some of the *L. barteri* fractions were more effective against the two strains of *A. fumigatus* than the test antifungal drug, fluconazole.

Tannins, polysaccharides, saponins, diterpenes, flavonoids, alkaloids, resins, glycosides, and steroids were found in the early phytochemical screening of methanol leaf extract and fractions of *L. barteri*. Flavonoids have been shown to minimize lipid peroxidation via enhancing vascularity as well as avoiding or reducing the initiation of cell necrosis [41]. As a result, any medicine that suppresses lipid peroxidation is thought to improve collagen fibril durability by improving collagen fibre strength, boosting perfusion, reducing cellular damage, and stimulating DNA transcription [42]. According to research, tannins [43,44], saponins [45,30] and triterpenoids [46] have also been shown to aid wound healing, owing to their astringent and antibacterial properties, which appear to be essential for wound contraction and higher epithelialization rates [47].

CONCLUSION

This study demonstrated the wound healing and antimicrobial activities of *L. barteri* leaf extract and fractions and it was found to be useful in the functional recovery of wound. The phytoconstituents present may have accelerated wound healing, thus, offering scientific proof to the ethnomedicinal applications of *L. barteri* leaves in wound therapy.

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