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Studies on the Antibacterial Potential and Time-kill Assays of Methanol Leaf Extract of *Lawsonia inermis* (Linn)

Olasunkanmi, Akintunde[1*], Olasunkanmi, Oluwaseun Oyetunji[2] and Akanbi, Oluwadamilola Eunice[3]

[1]Department of Science Laboratory Technology, Federal Polytechnic, Ede, Nigeria.
[2]Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria.
[3]Department of English Language, Obafemi Awolowo University, Ile-Ife, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author OA initiated the study, did literature search, wrote the manuscript and performed the statistical analysis. Author OOO designed the study, wrote the protocol and carried out the experiments. Author AOE proof read the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Lawsonia inermis is used for the treatment of several ailments such as rheumatoid arthritis, diarrhoea and skin diseases. The design of this study focused on the determination of the antibacterial potential and rate of killing of some bacterial isolates by methanol leaf extract of *Lawsonia inermis*. Agar-well diffusion method was used to determine the antibacterial activity of the extract obtained against a panel of bacteria. The minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), phytochemical and rate of killing of some bacterial isolates by the leaf extract were also studied. The presence of saponins, phytosterol, phenol,

**Corresponding author: E-mail: tjseun9@gmail.com;*

flavonoids, glycosides and alkaloids was revealed by phytochemical analysis. Appreciable antibacterial activity by the extract at a final concentration of 35 mg/mL was observed against all the bacterial strains used for this study. The mean diameter of zones of inhibition exhibited by the extract ranged between 9.0±1.0 mm - 17.3±0.6 mm while conventional ampicillin and streptomycin diameter of zones of inhibition ranged from 16.0±0.0 mm - 28.3±0.7 mm and 13.0±0.0 mm - 26.3±0.6 mm respectively. The MIC exhibited by the extract against susceptible test organisms ranged between 0.27 mg/mL - 4.375 mg/mL while MBC ranged between 0.55 mg/mL and 8.75 mg/mL. The time kill assay showed that the percentage of the cells killed increased with increasing concentrations of the extract, as well as, contact time intervals. In conclusion, the methanol leaf extract of *L. inermis* contains various phytochemicals which accounted for the appreciable antibacterial activity exhibited against some of the bacterial strains used in this study and thus supports its usefulness as a potential source of antibacterial agents.

Keywords: Antibacterial activity; Lawsonia inermis; phytochemical; rate of killing.

1. INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases and offer a new source of biologically active compound as antimicrobial agents. It has been estimated that 14-28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on ethno-medicinal use of the plants [1].

Lawsonia inermis which belongs to the family Lythraceae, is a perennial plant. It is known as Henna plant in English, *Lali* in Yoruba, *Laali* in Hausa, in India viz; *Mehndi* in Hindi, *Rakigarbha* in Sanskrit*, Mailanchi* in Malayalam, *Muruthani* in Tamil, *Benjati* in Oriya, *Mayilanchi* in kannada and *Mehedi* in Bengali. It originated in North Africa and Southeast Asia and is grown as an ornamental and dye plant [2].

It is much branched glabrous shrub or small tree with a usual height range of 2 - 6 m. The leaves are small, opposite in arrangement along the branches, sub-sessile, about 1.5 to 5 cm long, 0.5 to 2 cm wide, greenish brown to dull green, elliptic to broadly lancelet with entire margin, petiole short and glabrous and acute or obtuse apex with tapering base. The young branches are green in colour and become reddish when mature. The bark is greyish brown, unarmed when young but branches of older trees are spine tipped [3]. The flowers are small about 1 cm across, fragrant, white or rose coloured with four crumbled petals. Calyx is 0.2 cm tube and 0.3 cm spread lobes. Fruit is a small brown coloured round capsule. The fruit opens irregularly and splits into four sections at maturity and is many seeded. Seeds are about 3 mm across, numerous, smooth, pyramidal, hard, thick seed coat with brownish coloration [4].

Henna leaves are very popular natural dye to color hand, finger, nails and hair. The dye molecule, lawsone is the chief constituent of the plant; its highest concentration is detected in the petioles (0.5-1.5%). In folk medicine henna has been used as astringent, antihemorrhagic, intestinal antineoplastic, cardio-inhibitory, hypotensive, sedative and also as therapeutic against diarrhoea amoebiasis, headache, jaundice and leprosy [5]. Henna leaves are used to treat jaundice, skin diseases, venereal diseases, smallpox, spermatorrhoea [6]. Henna leaves, flowers, seeds, stem bark, roots are used agent to treat ailments such as rheumatoid arthritis, headache, ulcers, leprosy, fever, leucorrhea, diabetes, cardiac disease [7]. The major bio active component in Henna, lawsone is a naturally occurring naphthoquinone. Studies on antibacterial activities of the natural naphtoquinones have demonstrated that, they are active against Gram positive bacteria such as *Staphylococcus aureus, Enterococcus faecium and Bacillus subtilis* [6].

The aim of this study was to screen qualitatively for the phytochemical constituents and also to evaluate the *in vitro* antibacterial activity and rate of killing of some selected bacterial strains by methanolic leaf extract of *L. inermis* from Nigeria.

2. MATERIALS AND METHODS

2.1 Source of Microorganism

Microorganisms used for this study included typed cultures of National Collection of Industrial Bacteria (NCIB) and Locally Isolated Organisms

(LIO), some of which were Gram-positive bacteria (*Bacillus subtilis* (NCIB 3610), *Enterococcus faecalis* (NCIB 775), *Micrococcus luteus* (NCIB 196), *Bacillus stearothermophilus* (NCIB 8222), *Bacillus cereus* (NCIB 6344), *Staphylococcus aureus* (NCIB 8588), *Bacillus anthracis* (LIO), *Streptococcus pneumoniae* (LIO), *Pseudomonas flourescens* (LIO), *Bacillus subtilis* (NCIB 3610) and *Trueperella pyogenes* (LIO)) and some Gram-negative bacteria (*Escherichia coli* (NCIB 86), *Pseudomonas aeruginosa* (NCIB 950), *Klebsiella pneumoniae* (NCIB 418), *Proteus vulgaris* (LIO) and *Shigella* sp. (LIO)).

2.2 Preparation of Inoculum

Firstly, five colonies of each test bacteria used for this experiment were picked into nutrient broth (Rapid Labs Ltd) and incubated at 37°C for 18-24 h in an incubator. After 18 h of incubation, the test organisms were standardized to 0.5 McFarland standard [8].

2.3 Plant Specimen and Preparation

The leaves of *L. inermis* used for this experiment was collected from Moniya Area (7°32'04.8"N, 3°55'00.3"E), Ibadan, Oyo State, Nigeria and identified at Obafemi Awolowo University, Ife Herbarium. *Lawsonia inermis* leaf was dried in hot-air oven at 40°C until a constant weight of the sample was achieved and fine powder of the dried leaves were obtained by pulverizing. Exactly 1500 g of the powered sample was extracted in 2500 ml of methanol and this was left on the laboratory bench for 96 h with occasional agitation during this period. The mixture was later filtered into a sterile flask using glass wool and funnel. The filtrate collected was concentrated *in vacuo* in a rotary evaporator and then lyophilized. The resultant powder was kept in air-tight bottle and stored at 4°C in the refrigerator for further use [9].

2.4 Phytochemical Analysis of the Leaf Extract of *Lawsonia inermis*

Chemical tests were carried out on the methanol crude extract of *Lawsonia inermis* using standard procedures according to Trease and Evans [10] and Harborne [11] with some modifications to identify the constituents.

2.4.1 Test for tannins

The extract (2 ml) was added to 2 mL of distilled water, and then add $FeCl_3$ in drops. A brownish-green precipitate indicated the presence of tannins.

2.4.2 Test for saponins

The extract (2 ml) was dissolved with 2 mL of Benedict's reagent. Blue black precipitate indicated the presence of saponins.

2.4.3 Test for anthraquinones

The extract (2 ml) was boiled with 5 ml 10% HCl for two minutes in water bath. It is filtered and allowed to cool. Equal volume of chloroform was added to the filtrate and then three drops of 10% Ammonia were added to the mixture and heated. Formation of rose pink colour indicated the presence of anthraquinones.

2.4.4 Test for glycosides

Exactly 0.5 g of the extract was dissolved in 2 mL of glacial acetic acid containing 1 drop of 1% $FeCl_3$. This was under laid with 2 ml conc. H_2SO_4. A brown ring obtained at the interphase indicated the presence of a deoxy sugar, characteristics of cardiac glycoside. A violet ring may appear below the ring while in the acetic acid layer; a greenish ring may form just above the ring and gradually spreads throughout this layer.

2.4.5 Test for flavonoids

To indicate presence of flavonoids, 2 mL extract was treated with 2 mL of 10% lead acetate solution. Development of a yellowish green colour indicated the presence of flavonoids.

2.4.6 Test for alkaloids

The extract (2 ml) and 2 mL of Wagner's reagent were mixed. The development of a brown precipitate indicated the presence of alkaloids.

2.4.7 Test for phytosterol

Small quantity of extract (0.5 g) was dissolved in 5 ml of chloroform and two drops of acetic anhydride were added. The mixture was boiled and allowed to cool, then, 2 ml concentrated sulphuric acid was added. Formation of a bluish green solution confirmed the presence of phytosterol.

2.4.8 Test for phenol

The extract (0.5 g) was treated with 3 ml of 10% lead acetate solution. Presence of phenolic

compounds was indicated by a bulky white precipitate.

2.4.9 Test for steroid

Exactly 0.5 g of the extract was dissolved in 3 mL of $CHCl_3$ and filtered. To the filtrate was added 3 drops of concentrated H_2SO_4 to form a lower layer. A reddish brown colour was taken as positive for steroid ring.

2.5 Antibacterial Susceptibility Test

The crude extract was screened for antibacterial activity using agar-well diffusion method on Muller-Hinton agar medium (Biomark Ltd) as described by Akinpelu [12]. An inoculum of the test organism was added to 18 mL of sterile molten Muller-Hinton agar medium which had already been cooled down to 45°C. This was well mixed and poured into a sterile Petri dish and allowed to set. The required numbers of wells were bored into the medium using 6 mm sterile cork borer. The wells were made about 5 mm to the edge of the plate and labelled accordingly. The extract was reconstituted into solution (35 mg/mL concentration) with methanol (97.5% v/v)/sterile distilled water at 1:1 (v/v) and was filled up into the wells. Streptomycin and ampicillin were used as positive controls at a concentration of 1 mg/mL. The plates were allowed to stand for one hour and thereafter, the plates were incubated upright at 37°C for 24 h. The relative susceptibility of the test organisms to the extract as indicated by clear zones of growth inhibition around the wells was measured and recorded in millimetres.

2.6 Determination of Minimum Inhibitory Concentrations (MICs)

The MICs of the extract was done using method described by Akinpelu and Kolawole [13]. Two-fold dilutions of the plant extract were prepared and 2 mL of different concentration of the solution were added to 18 mL of pre-sterilized molten nutrient agar (Biomark Ltd) in McCartney bottle at a temperature of 45°C to a series of concentrations between 0.137 mg/mL and 35 mg/mL. The media were then poured into sterile Petri dishes and allowed to set. The surface of the nutrient agar plate was allowed to dry before streaking with standardized 18 h old broth culture of the susceptible bacterial strains. All the plates were then labelled accordingly and incubated at 37°C for 24 h and then examined for the presence or absence of growth of the test organisms. The lowest concentrations preventing the growth of the test organisms were taken as the minimum inhibitory concentrations (MICs) of the extract.

2.7 Determination of Minimum Bacte-ricidal Concentrations (MBCs)

Samples from plates with no apparent growth in the MIC assay plate were sub-cultured onto freshly prepared sterile nutrient agar plates without extract inclusion and then incubated at 37°C for 48 h. The lowest concentration of the extract that did not show any growth on a new set of nutrient plate was taken as the minimum bactericidal concentration of the extract [14].

2.8 Rate of Killing of *L. inermis* Methanolic Leaf Extract

The rate of kill was determined using the method described by Odenholt et al. [15] with some modifications. Experiment was performed using the extract on the viability of *E. coli* (NCIB 86) representing Gram-negative and *B. stearothermophilus* (NCIB 8222) representing Gram-positive organisms. Viable count of the test organisms was initially determined. A 0.5 mL volume of known cell density (by viable counts of 10^6 cfu/mL) from each test organism suspension was added to 4.5 mL of different concentration of the extract. The suspension was thoroughly mixed and held at room temperature (28-30°C) and the killing rate was determined over a period of 2 h. Exactly 0.5 mL of each suspension was withdrawn at the appropriate time intervals and transferred to 4.5 mL nutrient broth recovery medium containing 3% “Tween 80” to neutralize the carry-over effects of the antimicrobial compounds from the test suspensions. The suspension was shaken properly then serially diluted up to 10^{-5} in sterile physiological saline. Exactly 0.5 mL of the final dilution of the test organism was transfer into pre-sterile nutrient agar at 45ºC and plated out. The plates were allowed to set and incubated upside down at 37ºC for 72 h. Control experiment which was set up without the inclusion of antimicrobial agent (i.e every other things except the extract. The extract was reconstituted with methanol and sterile distilled water at 1:1). Viable counts were made in triplicates for each sample. Depression in the viable counts indicated killing by the antimicrobial agent.

3. RESULTS

The extract obtained from *L. inermis* leaves was brownish yellow in colour with a yield of 112 g

(7.47%) of extract from 1500 g of the powdered leaves of *L. inermis* that was extracted. The phytochemical analysis of the leaf extract of *L. inermis* revealed the presence of saponin, phytosterol, phenol, flavonoid, glycoside and alkaloid as presented in Table 1.

The extract of *L. inermis* at a concentration of 35 mg/mL was found to be active against all the bacterial strains used for this study with varying level of activity (Table 2). The zones of inhibition exhibited by the extract against the bacterial strains ranged between 9.0 mm and 17.3 mm, of which *Shigella* sp. had the lowest zone of inhibition of 9.0 mm while *Bacillus stearothermophilus* had the highest recorded zone of inhibition of 17.3 mm. The MIC value of 0.27 mg/mL was exhibited by the extract against *S. aureus, P. vulgaris* and *B. stearothermophilus*. *Pseudomonas aeruginosa, B. anthracis* and *M. luteus* had MIC value of 0.55 mg/mL, while that of *E. faecalis, E. coli, K. pneumoniae, P. flourescens* and *T. pyogenes* was 1.09 mg/mL (Table 3). Highest MIC value of 4.375 mg/mL was noted for *Shigella* sp.

The MBC exhibited by *L. inermis* methanolic leaf extract against *P. vulgaris, S. aureus* and *B. stearothermophilus* was 0.55 mg/mL. Minimum bactericidal concentration of 2.19 mg/mL was observed for *E. coli, E. faecalis, T. pyogenes* and *P. flourescens*, while that of *Shigella* sp. was the highest at 8.75 mg/mL.

The rate and extent of killing of *E. coli* and *B. stearothermophilus* cells by methanolic extract at 1 x MIC, 2 x MIC and 3 x MIC concentrations are as shown in Figs. 1 and 2 respectively. The percentage of the *E. coli* cells killed by the extract at 1 x MIC in 15 minutes was 4.1% while at 30 minutes, 13.4%. After 60 minutes of contact time with this extract, the percentage of cells killed was 23.7%. When the contact time was later increased to 90 minutes, 32.9% of the test organisms were killed and this rose to 51.7% after 120 minutes of contact time.

Table 1. Phytochemical components of *L. inermis* methanolic leaf extract

Phytochemical components	Result
Saponin	Present
Phytosterol	Present
Tannin	Absent
Phenol	Present
Steroid	Absent
Flavonoid	Present
Anthraquinone	Absent
Glycoside	Present
Alkaloid	Present

Table 2. Antibacterial activity of *L. inermis* methanolic leaf extract against some selected bacterial isolates

	Microorganisms	Zones of inhibition (mm)*			
		***L. inermis* (35 mg/mL)**	**Strep (1 mg/mL)**	**Amp (1 mg/mL)**	**Met/Wat (1:1) (v/v)**
Gram positive	*Bacillus anthracis* (LIO)	14.0±1.0	19.0±0.0	18.3±0.6	0
	Enterococcus faecalis (NCIB 775)	11.3±0.6	19.0±0.0	18.3±0.6	0
	Bacillus subtilis (NCIB 3610)	10.0±1.0	22.3±0.7	16.0±0.0	0
	Streptococcus pneumoniae (LIO)	10.0±0.0	23.3±0.7	19.7±0.6	0
	Micrococcus luteus (NCIB 196)	15.6±0.7	14.0±0.0	25.3±1.2	0
	Bacillus stearothermophilus (NCIB 8222)	17.3±0.6	20.0±0.0	28.3±0.7	0
	Staphylococcus aureus (NCIB 8588)	15.3±0.7	15.3±0.6	23.3±1.5	0
	Bacillus cereus (NCIB 6344)	11.7±0.6	13.3±0.7	17.0±1.0	0
	Trueperella pyogenes (LIO)	13.3±0.7	26.3±0.6	27.3±0.6	0
	Pseudomonas flourescens (LIO)	14.0±0.0	18.0±0.0	22.0±1.0	0
Gram negative	*Shigella* sp. (LIO)	9.0±1.0	13.0±0.0	20.0±1.0	0
	Klebsiella pneumoniae (NCIB 418)	10.3±1.2	21.0±1.0	21.6±0.7	0
	Proteus vulgaris (LIO)	14.3±0.7	20.0±1.0	29.3±1.5	0
	Escherichia coli (NCIB 86)	12.3±1.5	23.3±1.6	26.0±1.0	0
	Pseudomonas aeruginosa (NCIB 950)	14.0±1.0	31.0±1.0	21.0±0.0	0

For L. inermis and Strep P= 3.01552E-05< 0.05 (P=0.05)
For L. inermis and Amp P=3.51346E-08< 0.05 (P=0.05)
Keys: mm: Mean of three replicates, L. inermis: Lawsonia inermis, Strep: Streptomycin, Amp: Ampicillin, LIO: Locally Isolated Organism, NCIB: National Collection for Industrial Bacteria, G+: Gram positive, G-: Gram negative, Met/Wat: Methanol/Sterile distilled water*

Table 3. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) exhibited by *L. inermis* methanolic leaf extract against susceptible bacterial test organisms

	Microorganisms	MIC (mg/mL)	MBC (mg/mL)
Gram positive	*Bacillus anthracis* (LIO)	0.55	1.09
	Enterococcus faecalis (NCIB 775)	1.09	2.19
	Trueperella pyogenes (LIO)	1.09	2.19
	Micrococcus luteus (NCIB 196)	0.55	1.09
	Streptococcus pneumoniae (LIO)	2.19	4.38
	Bacillus cereus (NCIB 6344)	2.19	4.38
	Bacillus subtilis (NCIB 3610)	2.19	4.38
	Staphylococcus aureus (NCIB 8588)	0.27	0.55
	Bacillus stearothermophilus (NCIB 8222)	0.27	0.55
Gram negative	*Pseudomonas flourescens* (LIO)	1.09	2.19
	Shigella sp. (LIO)	4.375	8.75
	Klebsiella pneumoniae (NCIB 418)	1.09	2.19
	Escherichia coli (NCIB 86)	1.09	2.19
	Pseudomonas aeruginosa (NCIB 950)	0.55	1.09
	Proteus vulgaris (LIO)	0.27	0.55

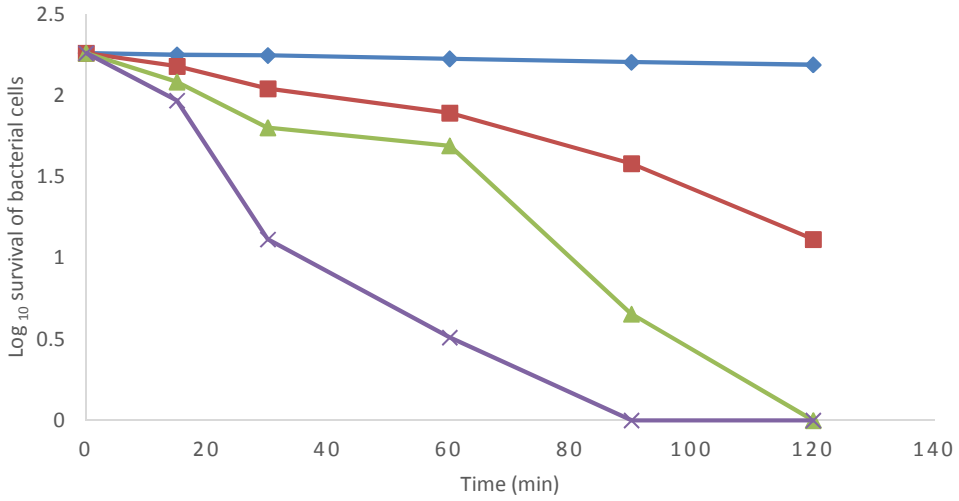

Fig. 1. The rate and extent of killing of *E. coli* cells by methanolic extract at 1 x MIC (—■—), 2 x MIC (—▲—), 3 x MIC (—✕—) and control (—◆—)
P= 0.012< 0.05 (P=0.05)

When the MIC of the extract was doubled, at exactly 15 minutes contact time, the percentage of the cells killed was 21.0%. At 30 minutes of contact time, 40.2% of the test cells were killed and when the contact time was increased to 60 minutes, the percentage of the cells killed was 53.1%. 79.8% of the total test cells were killed at 90 minutes, while 100% kill was achieved at 120 minutes of contact time. The extent and rate of killing of the test cells by the extract at 3 x MIC followed the same trend as the concentrations of the extract increased with increase in contact time, the percentage of the organisms killed also increased.

The percentage of the *B. stearothermophilus* killed by the extract at 1 x MIC in 15 minutes was 11.3% while the percentage of the cells killed at 30 minutes rose to 17.9%. After 60 minutes of contact time with this extract, the percentage of the test cells killed was 56.4%. When the contact time was later increased to 90 minutes, the percentage of the cells killed was 86.3% and this rose to 100% after 120 minutes of contact time. When the MIC of the extract was doubled, 100% cells kill was achieved within 90 minutes of contact time and at 3 x MIC, 100% cells kill was attained at 60 minutes of contact time.

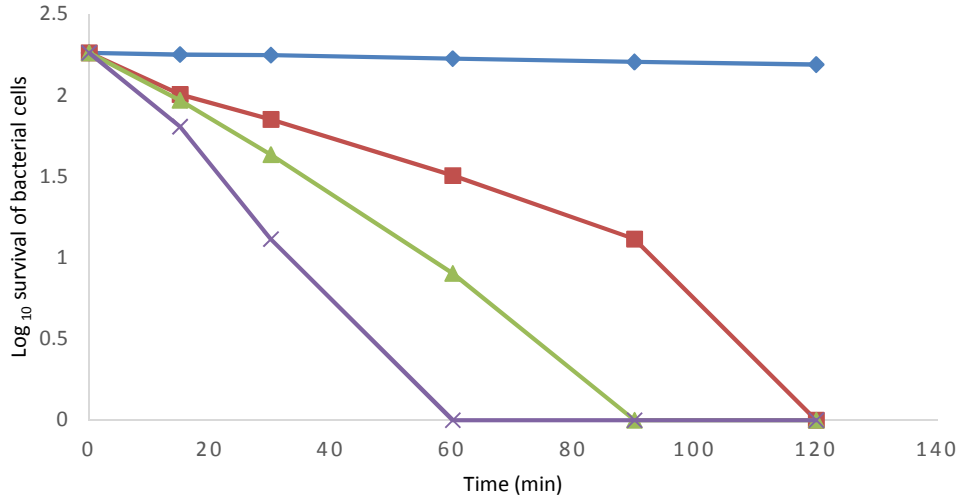

Fig. 2. The rate and extent of killing of *B. stearothermophilus* cells by methanolic extract at 1 x MIC (—■—), 2 x MIC (—▲—), 3 x MIC (—✕—) and control (—◆—)
P= 0.002< 0.05 (P=0.05)

4. DISCUSSION

The phytochemical analysis of the methanolic leaf extracts of *L. inermis* revealed the presence of saponins, phytosterol, phenol, flavonoids, glycosides and alkaloids which was slightly different from the report of Ali et al. [16]. Phytochemical components are known to be biologically active, contributing to antibacterial activity of medicinal plants. Saponins exhibit cytotoxic effect and growth inhibition against a variety of cell which make them have anti-inflammatory and anti-cancer properties as well as tumour inhibiting activity in animals [17]. Alkaloids have been studied for their potentials in elimination of human cancer cell lines [18], and are present in *L. inermis* as revealed in this study. Flavonoids found present in the leaf of *L. inermis* are known to possess antibacterial, anti-inflammatory, anti-allergic, anti-thrombotic, vasodilatory and anti-oxidant properties [19].

The result of this study shows that the extract exhibited *in vitro* antibacterial activity against all the bacterial isolates at a final concentration of 35 mg/mL. The highest zone of inhibition exhibited by the extract against *B. stearothermophilus* was 17.3±0.6 mm as against 17.0±16.04 mm reported by Kannahi and Vinotha [20] for *Streptococcus mutans*.

Kannahi and Vinotha [20] reported a zone of inhibition of 4.6±3.16 mm for *P. aeruginosa* which was far lesser than the 14.0±1.0 mm reported for this organism in this study. A zone of inhibition of 14.3±1.87 mm was reported by Ali et al. [16] for *S. aureus* different from 15.3±0.7 mm obtained in this study. Methanolic leaf extract of *L. inermis* had the highest antibacterial activity as reported by Ali et al. [16] and Kannahi and Vinotha [20] which this study also corroborated.

The value of the minimum inhibitory concentrations is usually an adequate guide for the treatment of most infections [21]. Methanolic leaf extract of this plant showed highest activity at MIC value of 0.27 mg/mL.

The methanolic leaf extract of *L. inermis* compared favourably with streptomycin and/or ampicillin used as standard control in this work as established in the level of activity and various zones of inhibition for the bacterial strains. Both Gram positive and Gram negative bacterial strains were susceptible to the extract thus an indication of broad spectrum activity.

The methanolic leaf extract of *L. inermis* exhibited appreciable kill rate against *B. stearothermophilus* and *E. coli.* As the concentrations of the extract increased with increase in contact time, there appeared to be an increase in populations of test cells killed. This is an indication of monophasic effect exhibited by the extract which was similar to the effect reported by Akinpelu et al. [22].

5. CONCLUSIONS

The leaf extract of *L. inermis* was found to contain saponins, phytosterol, phenol, flavonoids, glycosides and alkaloids. The extract also demonstrated a broad spectrum of antibacterial activity. There is the need to further explore the pharmacological potentials of this plant.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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