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Lycium shawii Roem. & Schult.: A new bioactive antimicrobial and antioxidant agent to combat multi-drug/pan-drug resistant pathogens of wound burn infections

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ABSTRACT

The Multidrug Drug Resistance (MDR) and Pan-Drug Resistance (PDR) remain an intractable challenge issue in public health, worldwide. Plant extracts-based biological macromolecules containing a diverse array of secondary metabolites could be potentially used as alternative approaches to control or limit MDR/PDR infections. Plants of the Solanaceae family exhibit a wide variety of secondary metabolites with antioxidant and antimicrobial properties, which render them a significant role in food and pharmaceutical applications. To our knowledge, this is the first report on phytochemical constituents, antioxidant, antimicrobial activities and *in vivo* toxicological safety of *Lycium shawii* leaf extracts. Results revealed that phenolics and flavonoids were found to be the most abundant compounds in all extracts. Antioxidant activity of extracts was measured using DPPH^{*} and ABTS^{•+} assays and the methanol extract displayed superior scavenging activity (IC₅₀ = 0.06 and 0.007 mg/mL for DPPH^{*} and ABTS^{•+}, respectively). Results of the GC-MS analysis revealed the identity of 10 compounds. Moreover, *in vivo* toxicological assessment can confirm the safety of *L. shawii* for use. Overall, *L. shawii* leaves are a promising natural source for the development of novel antimicrobial and antioxidant agents that could potentially combat clinical MDR/PDR pathogens.

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1. Introduction

In recent years, nosocomial infections by bacterial and fungal pathogens conferring Multi-Drug Resistance (MDR) or Pan-Drug Resistance (PDR) to various commercially available antibacterial and antifungal agents have become an intractable challenging issue worldwide. MDR is defined as resistance to three or more antimicrobial classes. However, resistance to all agents in all antimicrobial

classes is defined as PDR.¹ Several drug-resistant strains including *Pseudomonas aeruginosa* (Gram-negative bacteria), *Staphylococcus aureus* (Gram-positive bacteria), *Aspergillus niger* (filamentous fungi) and *Candida albicans* (unicellular fungi) have been known as most terrible pathogens of burn infections,^{1–8} to which effective alternative remedies are urgently required. Therefore, it is crucial to discover novel antimicrobial agents that can potentially in demand to combating drug resistance, as well as to treat these life-threatening infections.^{9,10}

Till date, biological properties and bioactive compounds of many medicinal plants are not studied.¹¹ Traditionally used medicinal plants produce a diverse array of bioactive phytoconstituents such as phenolics, alkaloids, flavonoids etc. These phytochemicals or phytochemicals derived from plant sources in the form of extracts displayed superiority over chemical approaches, and they have shown the potential to avert the danger caused by deadly

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Abbreviations			
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)	MBC	Minimum Bactericidal Concentration
AlCl ₃	Aluminum trichloride	MDR	Multi-Drug Resistance
DMSO	Dimethyl sulfoxide	MFC	Minimum Fungicidal Concentration
DIZ	Diameter Inhibition Zone	MIC	Minimum Inhibitory Concentration
DPPH	1,1-diphenyl-2-picrylhydrazyl	NIST	National Institute of Standard and Technology
GAE	Gallic Acid Equivalent	OECD	Organization for Economic Cooperation and Development
GC-MS	Gas Chromatography-Mass Spectrum	PBP	Penicillin-Binding Protein
GSH	Glutathione	PDR	Pan-Drug Resistance
HPLC	High Performance Liquid Chromatography	QE	Quercetin
IAEC	Institutional Animal Ethics Committee	TEM	Transmission Electron Microscopy
LD ₅₀	Lethal Dose 50	TFC	Total Flavonoid Content
		TPC	Total Phenolic Content

infections.^{12–14} Hamidpour et al.¹⁵ reported that the Russian olive (*Elaeagnus angustifolia* L.) extract has novel roles as active antioxidant, anti-inflammatory and analgesic agent. This extract has shown to be effective in reducing the healing time of wounds in injured patients. The leaf extract of *Alpinia nigra* possesses profound antibacterial, analgesic and cytotoxic activities.¹⁴ Heidari et al.¹³ reported the efficacy of topical application of a standardized extract of *Tragopogon graminifolius* in the healing process of burn wounds. They concluded that this extract could be considered as a future treatment for healing of burn wounds. Many commercially established antibiotics incorporated in the present-day use medicine has been suggested potentially useful biological activity.¹⁶ Interestingly, the principle advantages of employing plant-based antimicrobials are that they are relatively safer and affordable than synthetic alternatives.^{17–21}

Wang et al.²² have proposed several mechanisms of antibacterial and antifungal potentials of ethnomedicinal plants, which include decomposition of the cytoplasmic membrane, disturbance of outer membrane of Gram-negative bacteria, accretion of ATPase, inhibition of enzymes synthesis, and coagulation of cell inner contents. It is reported that Gram-negative bacteria are more resistant towards the antibacterial activities of medicinal plant extracts as compared to Gram-positive bacteria and this is attributed to the presence of outer membrane structure in Gram-negative cells that reduced the penetration rate of compounds with antimicrobial activities.^{9,10} On the other hand, the mechanisms of antifungal actions are consonant to those described above for bacteria, including irreversible trauma to the cell septum, exuding and coagulation of cellular interior materials.²³

Plants of the Solanaceae family exhibit a wide variety of secondary metabolites with different biological activities including nutritional antioxidants, antimicrobials, anti-inflammatory, anti-cancer or cytotoxic properties, which render them to play significant roles in food and pharmaceutical applications.^{24–26} Recent phytochemical studies confirmed that the richness of numerous constituents, such as terpenoids, alkaloids and flavonoids endows *Lycium* species (Solanaceae family) with a variety of biological activities.¹⁶ The fruits, flowers and roots of many *Lycium* species have long been used as an important source of food and/or medicine.²⁷ However, to the best of the authors' knowledge, to date, there have been no reports to investigate the leaf extract of *Lycium shawii* as an alternative source of natural antioxidant and antimicrobial agent combating MDR/PDR pathogens of burn wound infections. Therefore, this study was aimed at evaluating the novel pharmaceutical potentials of the traditional medicinal plant, *L. shawii*, as active antimicrobial against MDR/PDR clinical pathogens of burn wound infections, antioxidant agent and with its toxicological

safety using acute oral toxicity and haemolytic activity for completeness of its assessment as a new leading structure in the pharmaceutical field.

2. Materials and methods

2.1. Plant collection

Egyptian *Lycium shawii* Roem. & Schult. leaves were collected from El-Omayed Biosphere Reserve area at the Northern Mediterranean Coast of Egypt (Supplemental information, Fig. S1). Table 1 depicts the botanical name, Arabic name, family, habit, habitat, distribution and medical uses of *L. shawii*.¹⁶ The leaves were washed with tap water, dried and pulverized to a fine powder by using a lab grinder. The resultant powder was stored for further experiments.

2.2. Crude extraction

A total of 50 g of dried *L. shawii* leaves was extracted with methanol using Soxhlet apparatus for 5–7 h at 50 °C. The solvent used for extraction was evaporated using rotary evaporator under reduced pressure. The crude extract slurry formed was thoroughly dried and weighted. Ethanol, ethyl acetate and aqueous (water) extracts were similarly performed. All extracts were freeze-dried at –20 °C and stored at 4 °C until use.

2.3. Phytochemical screening

Phytochemical components (flavonoids, alkaloids, phenolics, tannins, saponins, steroids, coumarins, glycosides and terpenoids) were screened in the four extracts of *L. shawii* leaves using standard conventional protocols.²⁸

2.4. Quantitative assay of total bioactive components

Phenolics and flavonoids were found to be the most abundant compounds in *L. shawii* extracts. Therefore, Total Flavonoid Content (TFC) and Total Phenolic Content (TPC) were determined as described below.

2.4.1. Total Flavonoid Content (TFC)

A total of 500 µL sample solution was mixed with the same volume of aluminum trichloride (AlCl₃; 2%) in methanol. A blank was prepared by adding a sample solution to methanol without AlCl₃. Similarly, the procedures were repeated for other *L. shawii* extracts. After a 10 min incubation at room temperature, blank and

Table 1
Ethnobotanical value of *Lycium shawii* used in this study.

Botanical name	Arabic name	Family	Habit	Habitat	Distribution	Medicinal uses	Other uses	Reference
<i>Lycium shawii</i> Roem. & Schult.	Awsaj	Solanaceae	Shrub	Sandy and silty depressions, runnels, wadis and on rocky slopes	Recorded in Egypt, Qatar, Bahrain, Kuwait, Saudi Arabia and UAE	A diuretic, laxative and tonic agent	Grazed by animals and the berries are eaten by man; the leaves provide browsing for camels	16

sample solutions were measured at 415 nm using a UV–visible spectrophotometer (Shimadzu-UV2600, Japan). TFC was expressed as milligrams of quercetin (QE) equivalents (mg QE/g extract).

2.4.2. Total Phenolic Content (TPC)

A total of 250 μ L sample solution was mixed with 1000 μ L Folin–Ciocalteu reagent (1:9; v/v) and the mixture was incubated for 3 min. Then, the Na_2CO_3 solution (750 μ L; 1%) was added. After a 2 h incubation at room temperature, the sample absorbance was measured at 760 nm using a UV–visible spectrophotometer (Shimadzu-UV2600, Japan) with a constructed calibration curve using gallic acid. TPC was expressed as milligrams of Gallic Acid Equivalents (mg GAE/g extract).

2.5. Antioxidant activity

Phenolic and flavonoid compounds are associated with antioxidant activity. Therefore, it is important to assess the antioxidant activity of *L. shawii* leaf extracts, and this assessment was performed as described below.

2.5.1. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH \cdot) assay

The scavenging activity against DPPH \cdot was estimated.²⁹ The assay depends on the reduction of DPPH \cdot (purple color) to a diphenyl picrylhydrazine (yellow color) and the remaining DPPH \cdot was measured at 517 nm after a 30 min incubation at 37 $^\circ$ C in the dark. Glutathione (GSH) was used as a positive control. The DPPH \cdot scavenging activity was expressed as milligrams of GSH equivalents (mg GSH/g extract).

2.5.2. 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS \cdot^+) assay

The scavenging activity against ABTS \cdot^+ was estimated.²⁹ The sample absorbance was measured at 734 nm after a 30 min incubation at 37 $^\circ$ C. The 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma, USA) was used as a positive control. The ABTS \cdot^+ scavenging activity was expressed as milligrams of Trolox equivalents (mg Trolox/g extract).

2.6. Microbial strains

Eleven strains of *Pseudomonas aeruginosa*,¹ 24 strains of *Staphylococcus aureus*,² 11 strains of *Aspergillus niger*,¹ and 12 strains of *Candida albicans*³⁰ used in this study were isolated previously from wound burn infections. As we previously described in details these strains were identified as MDR or PDR strains.^{1,2,30}

2.7. Antimicrobial activity, Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

Preliminary screening of antimicrobial activities of the four *L. shawii* extracts was performed against 58 drug-resistant strains of bacteria and fungi (data not shown) using the disc diffusion method as we previously described in details.¹ According to our

results, four strains; namely *P. aeruginosa* 9 (PA-09), *S. aureus* 17 (SA-17), *A. niger* 5 (AN-05) and *C. albicans* 11 (CA-11) were selected for further experiments. *L. shawii* extract was dissolved in pure dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri, USA) to a final concentration of 100 mg plant extract in 1 mL of DMSO. The extract was sterilized by filtration on 0.45 μ m Millipore filters. Then, 20 μ L of this extract was soaked into sterile filter paper discs. These discs were placed on Muller-Hinton agar plates previously swabbed with 100 μ L of bacterial and fungal inoculum (approximately 10^8 CFU/mL). The DMSO was used as a negative control. The MIC and MFC of *L. shawii* leaf extracts were performed by a serial dilution technique.³¹ MIC and MFC were regarded as the lowest concentration *L. shawii* extract at which no change the color occurred.

2.8. Transmission Electron Microscopy (TEM)

The mechanism of antimicrobial action of the *L. shawii* methanol extract was performed using TEM as we previously described in details.^{3,6,32} Briefly, DMSO-treated cells (control), cells incubated without DMSO (control), and cells treated with the MIC of *L. shawii* extract were prepared. Samples were examined using a TEM (JEOL-JEM-100SX, Japan).

2.9. Gas Chromatography-Mass Spectrum (GC-MS) analysis

The methanol extract of *L. shawii* was concentrated in a desiccator and subjected to GC-MS analysis. GC-MS model Agilent 8790B/5977B MSD was used in the analysis that employs Agilent 19091s-433UI Hp-5 column (60–325 $^\circ$ C) and the components were separated using helium (He) as a carrier gas at a constant flow of 0.8 mL/min at ionization energy (70eV). The sample extract (1 μ L) was injected into the instrument. The injector temperature was set at 250 $^\circ$ C and throughout the analysis, temperature flow was set at the speed of increasing 10 $^\circ$ C/min. The name, molecular weight, compound nature and a molecular formula of the test sample were identified based on the National Institute of Standard and Technology (NIST) library spectra data bases.

2.10. Toxicological assessment

2.10.1. Experimental animals

Thirty albino rats (170–200 g/rat) were used as an experimental animal model for the *in vivo* oral toxicity assessment of the methanol extract of *L. shawii* leaves. The rats were kept for seven days prior to oral administration at the Animal House of Faculty of Science, Tanta University, Egypt to allow for their acclimatization to the laboratory environmental conditions as we previously described in details.^{1,4} The animal experimental protocols were performed in accordance with the requirements of the Institutional Animal Ethics Committee (IAEC) guidelines and approved by IAEC, Faculty of Science, Tanta University, Egypt (code; Rec-Sci-Tu-020).

2.10.2. Acute oral toxicity

The toxicity test was designed to obtain the lethal dose 50 (LD₅₀)

of *L. shawii* leaf extract according to the Organization for Economic Cooperation and Development (OECD) Guideline.³³ To determine the LD₅₀, rats divided into five groups, three rats in each. The control group was administered with 0.5 mL saline solution, while other four groups were administered with methanol extract concentrations (1000–4000 mg/kg body weight) orally by gavage. Both control and treated groups were administered once daily for two weeks. Visual observations including external appearance, body weights, and daily activities were monitored throughout the experimental period.

2.10.3. Haemolytic activity

The haemolytic activity assay of *L. shawii* extract was determined as previously described.¹⁰ Briefly, the red blood cells were collected from the healthy albino rats and the blood cell suspension was prepared by diluting the cell pellet to 0.5% with phosphate-buffered saline. Free haemoglobin of the supernatant was measured at 405 nm using a UV–visible spectrophotometer (Shimadzu-UV2600, Japan). Three replicates were done at each extract concentration. Haemolytic activity was depicted by an increase in the optical density of the solution due to the release of hemoglobin through time.

2.11. Statistical analysis

Results are presented as the mean ± standard deviation of three replicates. The statistical analyses were carried out using SPSS-20. The obtained data were analyzed statistically to determine the degree of significance using one-way analysis of variance and Student's t-test at a probability level ≤0.05. The Inhibitory Concentration 50 (IC₅₀) values were calculated by linear regression analysis. Linear regression was analyzed by Origin 6.0 professional software.

3. Results and discussion

3.1. Phytochemical screening of the *L. shawii* extracts

It is not enough to make a true assessment of the bioactivity potential of a plant without considering its chemistry. The naturally occurring bioactive compounds are considered to be crucial for the biological activities of any plant species. Taken together, it is

necessary to reveal the biological activity and the chemical composition as well as the safety assessment of the extracts from the Egyptian *L. shawii* leaves. As depicted in Table 2, the aqueous, methanol, ethanol and ethyl acetate extracts of *L. shawii* leaves were evaluated for their phytochemical composition including alkaloids, phenolics, flavonoids, tannins, saponins, glycosides, terpenoids, steroids and coumarins. The results revealed that phenolics and flavonoids were found to be the most abundant compounds in all extracts tested. On the other hand, saponins and coumarins were absent from all extracts. The high incidence of phytochemical compounds detected resulted in the superiority of the methanol extract than that of the ethanol, ethyl acetate and aqueous extracts, respectively. Most of the conventional antibiotics often fail to curb the proliferation of MDR/PDR clinical pathogens of bacteria and fungi in wound burn infections making the treatment of burns a difficult task. Plant extracts contain a diversity of phytochemical compounds that used for the treatment of wound burn infections since times immemorial. They also have a wide range of medicinal properties such as antibacterial, antifungal, antioxidant, anti-inflammatory, antiallergic, antiproliferative, antidiabetic, antiviral and analgesic that may prevent the development of drug-resistant bacteria and fungi as reported for many traditional plant extracts.^{34,35} However, the information of *L. shawii* is limited. To the best of the authors' knowledge there have been no reports on the biological activities and the chemical composition of *L. shawii* leaves. Therefore, this study was aimed to assess the antimicrobial and antioxidant activities of *L. shawii* leaves with their toxicological evaluation.

3.2. Total bioactive compounds

As shown in Table 2, phenolics and flavonoids were found to be the most abundant phytochemical compounds in the *L. shawii* extracts. Therefore, total quantities of these compounds were determined and results are given in Table 3. Phenolic compounds are associated with antioxidant activity. Hence, it is important to quantify TPC and to assess its contribution to antioxidant activity. Clearly, the antioxidant activity of phenolic compounds is largely associated with their redox potential which give them the superiority to act as potential metal chelators, hydrogen donors, reducing agents as well as single oxygen quenchers.³⁶ Our results of TPC showed their wide variation in the four extracts tested, ranging

Table 2
Screening of phytochemical constituents of *L. shawii* leaf extracts.

Phytochemical constituents	Solvent extract				Test or reagent used	Observed color	Uses	Mechanism of action	References
	Aqueous	Methanol	Ethanol	Ethyl acetate					
Alkaloids	ND	+	+	ND	Wagner	Pale yellow	Antibacterial, anti-inflammatory, analgesic	Intercalate into DNA and uncouple respiration	34
Flavonoids	+	+	+	+	AlCl ₃	Yellow	Antimicrobial, diuretic	Metal chelating, cell wall complexation, adhesion binding	35
Phenolics	+	+	+	+	AlCl ₃	Yellow	Antimicrobial, wound dressing	Membrane infraction	35
Tannins	ND	+	+	+	Braemers	Dark blue	Antimicrobial, wound dressing, anti-inflammatory, preventing ulcer development	Enzyme inactivation, cell wall complexation, adhesion binding, membrane disruption, substrate deprivation	34
Saponins	ND	ND	ND	ND		ND	–	–	
Glycosides	ND	+	ND	+	H ₂ SO ₄	Dark brown	Lower blood pressure	NA	
Terpenoids	ND	+	+	+	Salkowaski	Red	Antimicrobial	Membrane disruption, prohibition of ATase actuality	35
Steroids	ND	+	+	ND	Salkowaski	Red	Antihypertensive effect on blood pressure, serum analysis of hypertensive patient	NA	
Coumarins	ND	ND	ND	ND		ND	–	–	

ND, Not Detected; NA, Not Available; +, present; -, absent; AlCl₃, aluminum trichloride; H₂SO₄, sulfuric acid.

Table 3Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of the solvent extract from *L. shawii* leaves.

Assay	Solvent extract				P-value
	Aqueous	Methanol	Ethanol	Ethyl acetate	
TFC (mg QE/g extract) ^I	95.93 ± 0.64 ^a	90.57 ± 0.31 ^b	41.47 ± 0.51 ^c	52.43 ± 0.98 ^d	< 0.001*
TPC (mg GAE/g extract) ^{II}	55.50 ± 1.18 ^a	230.50 ± 0.50 ^b	99.93 ± 0.83 ^c	190.60 ± 0.40 ^d	< 0.001*

Different superscript letters in the same row indicate significant difference ($P \leq 0.05$); ^I QE, Quercetin Equivalent; ^{II} GAE, Gallic Acid Equivalent.

from 55.50 ± 1.18 to 230.50 ± 0.50 mg GAE/g extract (Table 3). According to these results, it is clear that the TPC depends on the type of extracted solvent used. The methanol extract had a significantly higher concentration of phenolics than ethyl acetate, ethanol and aqueous extracts, respectively. This variation may be due to the phenolics polarity. Additionally, our findings may explain the widespread uses of the folkloric plants. On the other hand, the TFC of the *L. shawii* extracts varied from 41.47 ± 0.51 to 95.93 ± 0.64 mg QE/g extract (Table 3). The assay of TFC was resulted in the superiority of the aqueous extract compared to the other extracts including methanol, ethyl acetate and ethanol, respectively. Flavonoids, as a major class of phenolic compounds, are exhibited a strong antioxidant activity. Clearly, our data of TPC and TFC suggest

L. shawii leaf extracts as a remarkable source of antioxidant components. Accordingly, the antioxidant activities of these leaf extracts are investigated in this study; especially there is no reports on the antioxidant activity of *L. shawii* leaves.

3.3. Antioxidant activity

The DPPH[•] and ABTS^{•+} assays have been widely used to determine the free radical-scavenging activity of various plant extracts. Both the DPPH[•] (Fig. 1A) and ABTS^{•+} (Fig. 1B) inhibition percentage values were dose-dependent, whereby they increased in the range of the tested concentrations, for the *L. shawii* extracts and the positive controls (GSH and Trolox for DPPH[•] and ABTS^{•+},

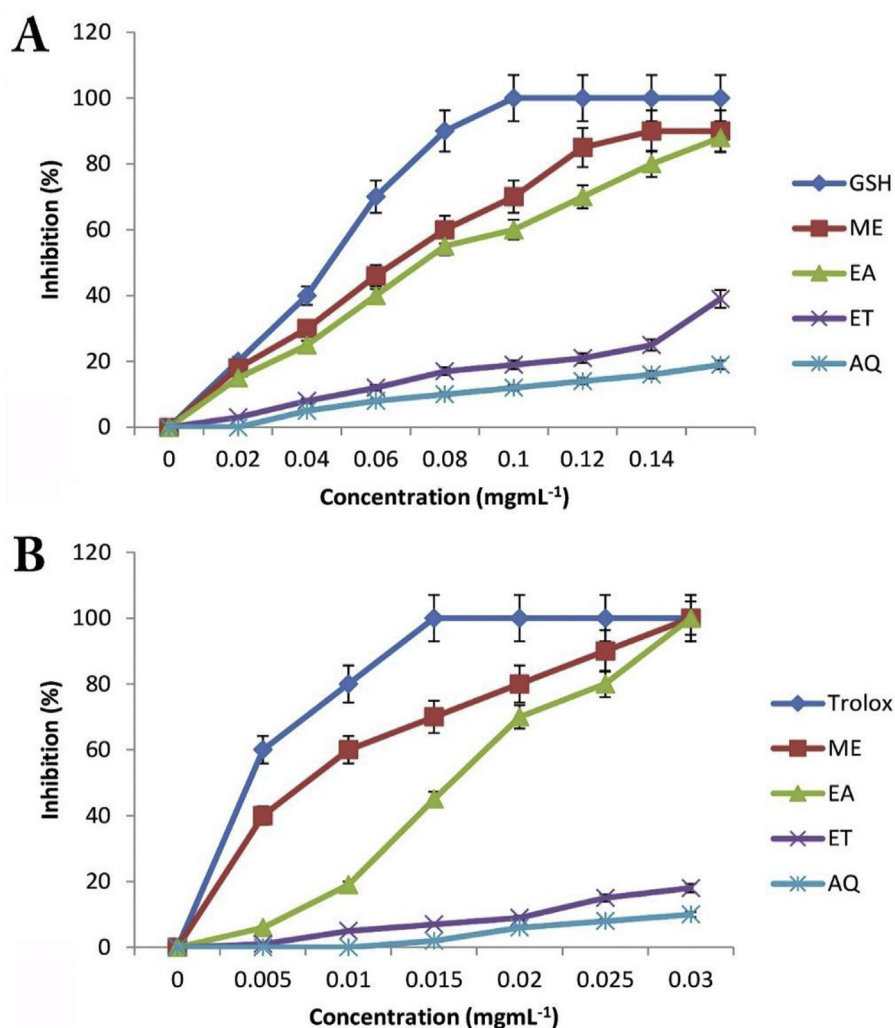


Fig. 1. Effect of different concentrations of *L. shawii* leaf extracts in free radical-scavenging activities: (A) DPPH[•] assay and (B) ABTS^{•+} assay. GSH: glutathione; GSH and Trolox are positive controls; ME, Methanol; EA, Ethyl Acetate; ET, Ethanol; AQ, Aqueous.

Table 4
DPPH• and ABTS•+ scavenging activities of leaf extracts from the *L. shawii*.

Radical scavenging activity		Solvent extract				Positive control	
		Aqueous	Methanol	Ethanol	Ethyl acetate	GSH	Trolox
DPPH•	IC ₅₀ (mgmL ⁻¹)	0.7	0.06	0.1	0.07	0.005	ND
	1/IC ₅₀	1.4	16.7	10.0	14.3	200	ND
ABTS•+	IC ₅₀ (mgmL ⁻¹)	0.15	0.007	0.075	0.015	ND	0.0025
	1/IC ₅₀	6.6	142.8	13.3	66.7	ND	400

ND: Not Detected; IC: Inhibitory Concentration; IC₅₀, the concentration required to inhibit 50% of radical.

respectively). Both the DPPH• and ABTS•+ inhibition increased in the extracts of aqueous, ethyl acetate, ethanol and methanol, respectively compared with positive controls used. The DPPH• and ABTS•+ scavenging activities (IC₅₀ values) of *L. shawii* leaf extracts were calculated and the results are presented in Table 4. In DPPH• and ABTS•+ assays, the methanol extract displayed superior scavenging activity (IC₅₀ = 0.06 and 0.007 mg/mL, respectively) compared to the other leaf extracts tested (Table 4). This can be attributed to the higher total phenolic and flavonoid contents. Similar antioxidant activities for other plant species by DPPH• and ABTS•+ assays have been reported.^{37,38} On the other hand, the aqueous extract of *L. shawii* showed the lowest scavenging activity in both assays (Table 4).

The correlation graphs between antioxidant activity (DPPH• and ABTS•+) and the total bioactive compound (TPC and TFC) of the four *L. shawii* extracts are presented in Fig. 2. A significant linear correlation appeared between the DPPH• scavenging activity and the TPC (Fig. 2A) and TFC (Fig. 2B) with an excellent correlation coefficient ($R^2 = 0.8997$ for TPC and $R^2 = 0.8553$ for TFC). Also, a significant linear correlation appeared between the ABTS•+ scavenging activity and the TPC (Fig. 2C) and TFC (Fig. 2D) with an excellent correlation coefficient ($R^2 = 0.9118$ for TPC and $R^2 = 0.8757$ for TFC). In agreement with previous reports,³⁹ a strong correlation between TPC and the antioxidant activity ($R^2 = 0.929$

and 0.814 for *Sidastrum micranthum* and *Wissadula perplicifolia* extracts, respectively). Therefore, the assessment of *L. shawii* extracts suggests that TPC and TFC of these extracts are largely responsible for the antioxidant activity, and our findings are in agreement with previous studies.⁴⁰ In conclusion, *L. shawii* leaves are a remarkable source of phenolics and flavonoids as well as antioxidant activity, therefore *L. shawii* leaves can be used as a novel antimicrobial agent against several infections due to the increased production of free radicals. Accordingly, safety assessment of the antimicrobial activity of *L. shawii* leaf extracts is needed to confirm the potential use of this plant species in disease treatment.

3.4. Antimicrobial activity

In the present study the antimicrobial activity of *L. shawii* was performed. Theoretically, Diameter Inhibition Zone (DIZ) reflects the antimicrobial concentration of the leaf extract tested. As presented in Table 5, the results show that the methanol extract (50 mg/mL) had the most detrimental effect against the MDR/PDR strains isolated from wound burn infections. According to our results, increasing the concentration of *L. shawii* extracts led to the DIZ to increase significantly ($P \leq 0.05$). In general, the antimicrobial activity of each solvent extraction varied according to the kind of the microbial pathogen. Clearly, the Gram-positive bacterial strain

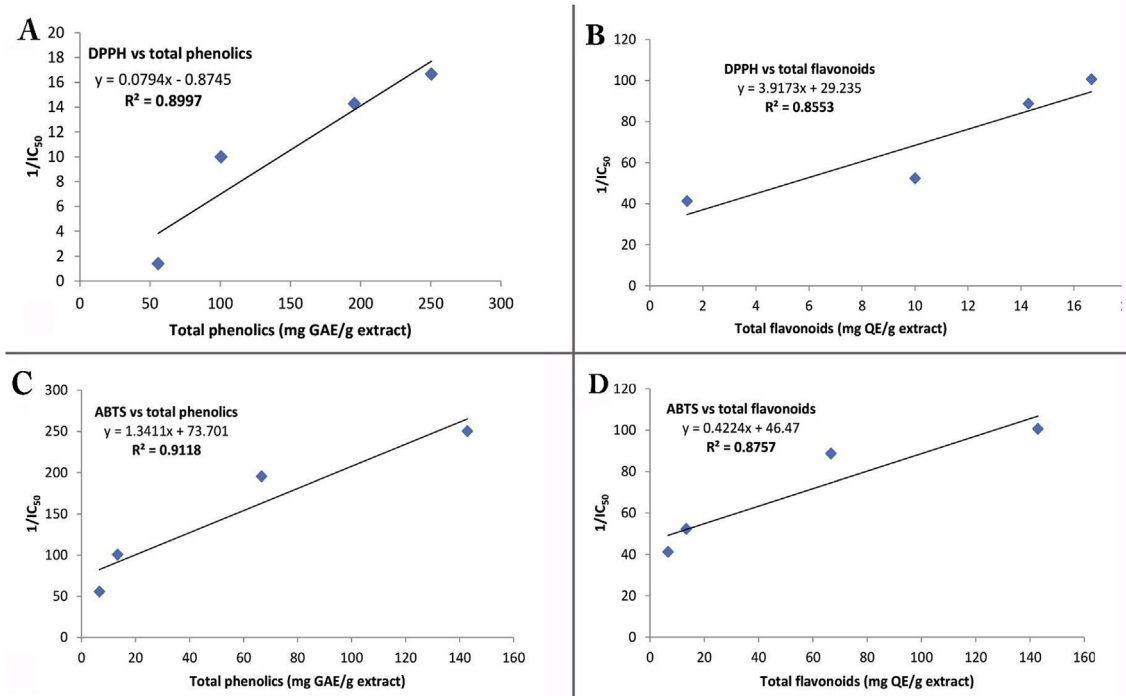


Fig. 2. Correlation coefficients graphs (R-values) between antioxidant activities (1/IC₅₀) and TPC (A&C) and TFC (B&D) using DPPH• assay (A&B) and ABTS•+ assay (C&D). QE, Quercetin Equivalent; GAE, Gallic Acid Equivalent.

Table 5
Antimicrobial activity of four extracts from *L. shawii* leaves.

Strain number	Extract	DIZ (mm)					MIC ($\mu\text{g mL}^{-1}$)	MBC ($\mu\text{g mL}^{-1}$)	MFC ($\mu\text{g mL}^{-1}$)	DRPs	Resistance type	
		Concentration of <i>L. shawii</i> leaf extract (mg mL^{-1})										
		10	20	30	40	50						
PA-09	ANOVA	AQ	0.0 ± 0.0	10.5 ± 0.1	13.7 ± 0.1	15.5 ± 0.1	15.9 ± 0.06	32	64	ND	AX, CAZ, FEP, ATM, CRO, IPM, CIP, SXT, C, CN, TOB, K, CT ¹	MDR
		ME	10.5 ± 0.1	14.6 ± 0.1	20.1 ± 0.1	25.3 ± 0.06	28.3 ± 0.1	4	8	ND		
		ET	8.7 ± 0.06	11.9 ± 0.1	14.7 ± 0.06	18.5 ± 0.06	20.00 ± 0.00	8	16	ND		
		EA	0.0 ± 0.0	9.3 ± 0.06	13.03 ± 0.06	16.9 ± 0.06	22.8 ± 0.15	16	32	ND		
		F	22353.867	1888.800	4667.458	6725.733	8039.639					
		P-value	*0.00	*0.00	*0.00	*0.00	*0.00					
SA-17	ANOVA	AQ	0.0 ± 0.0	9.3 ± 0.06	12.0 ± 0.0	16.1 ± 0.06	19.3 ± 0.1	16	32	ND	AX, PIP, AMC, TZP, CTX, CRO, FEP, CFP, IMP, ATM, NA, CIP, CN, AK, K, SXT, TE, C, VA ²	PDR
		ME	13.2 ± 0.06	18.0 ± 0.0	23.5 ± 0.06	30.3 ± 0.1	34.9 ± 0.06	2	4	ND		
		ET	8.03 ± 0.06	10.6 ± 0.1	13.8 ± 0.1	19.0 ± 0.0	22.1 ± 0.1	8	16	ND		
		EA	7.9 ± 0.06	11.03 ± 0.06	15.4 ± 0.1	19.7 ± 0.06	23.03 ± 0.06	8	16	ND		
		F	35458.222	11079.133	8399.848	15564.296	13282.333					
		P-value	*0.00	*0.00	*0.00	*0.00	*0.00					
CA-11	ANOVA	AQ	0.0 ± 0.0	0.0 ± 0.0	10.03 ± 0.06	12.5 ± 0.06	15.0 ± 0.0	16	ND	64	AMB, ITC, CLT, MIZ, FLC, MCFG, NYT, TRB ⁹	PDR
		ME	8.03 ± 0.06	15.03 ± 0.06	28.4 ± 0.06	40.00 ± 0.0	50.8 ± 0.06	4	ND	8		
		ET	0.0 ± 0.0	9.1 ± 0.06	15.2 ± 0.06	21.5 ± 0.06	24.7 ± 0.1	8	ND	8		
		EA	0.0 ± 0.0	0.0 ± 0.0	11.00 ± 0.00	18.23 ± 0.06	22.77 ± 0.12	8	ND	16		
		F	58081.000	48962.000	86313.222	169443.889	109232.458					
		P-value	*0.00	*0.00	*0.00	*0.00	*0.00					
AN-05	ANOVA	AQ	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.2 ± 0.2	10.6 ± 0.1	32	ND	64	AMB, ITC, CLT, MIZ, FLC, TRB ¹	MDR
		ME	10.0 ± 0.0	22.1 ± 0.1	36.7 ± 0.06	49.4 ± 0.1	54.0 ± 0.0	8	ND	16		
		ET	8.0 ± 0.0	22.2 ± 19.05	20.0 ± 0.0	32.03 ± 0.06	34.1 ± 0.06	16	ND	16		
		EA	8.03 ± 0.06	11.6 ± 0.1	24.6 ± 0.2	35.8 ± 0.1	39.5 ± 0.06	16	ND	32		
		F	70921.000	3.690	84086.400	32355.226	195569.944					
		P-value	*0.00	*0.00	*0.00	*0.00	*0.00					

PA, *Pseudomonas aeruginosa*; **SA**, *Staphylococcus aureus*; **CA**, *Candida albicans*; **AN**: *Aspergillus niger*; **AQ**, Aqueous; **ME**, Methanol; **ET**, Ethanol; **EA**, Ethyl Acetate; **MIC**, Minimum Inhibitory Concentration; **MBC**, Minimum Bactericidal Concentration; **MFC**, Minimum Fungicidal Concentration; **DRPs**, Drug-Resistance Patterns; **MDR**, Multi-Drug Resistance; **PDR**, Pan-Drug Resistance; **AX**, amoxicillin; **CAZ**, ceftazidime; **FEP**, cefepime; **ATM**, aztreonam, **CRO**, ceftriaxone; **IMP**, imipenem; **CIP**, ciprofloxacin; **SXT**, cotrimoxazole; **C**, chloramphenicol; **CN**, gentamicin; **TOB**, tobramycin; **K**, kanamycin; **CT**, colistin sulfate; **PIP**, piperacillin; **AMC**, amoxicillin/clavulanic acid; **TZP**, piperacillin/tazobactam; **CTX**, cefotaxime; **CFP**, cefoperazone; **NA**, nalidixic acid; **AK**, amikacin; **TE**, tetracycline; **VA**, vancomycin; **AMB**, amphotericin; **FLC**, fluconazole; **ITC**, itraconazole; **CLT**, clotrimazole; **MIZ**, miconazole; **MCFG**, micfungin; **NYT**, nystatin; **TRB**, terbinafine; **ND**, Not Detected; **P*-value ≤ 0.05 is significant.

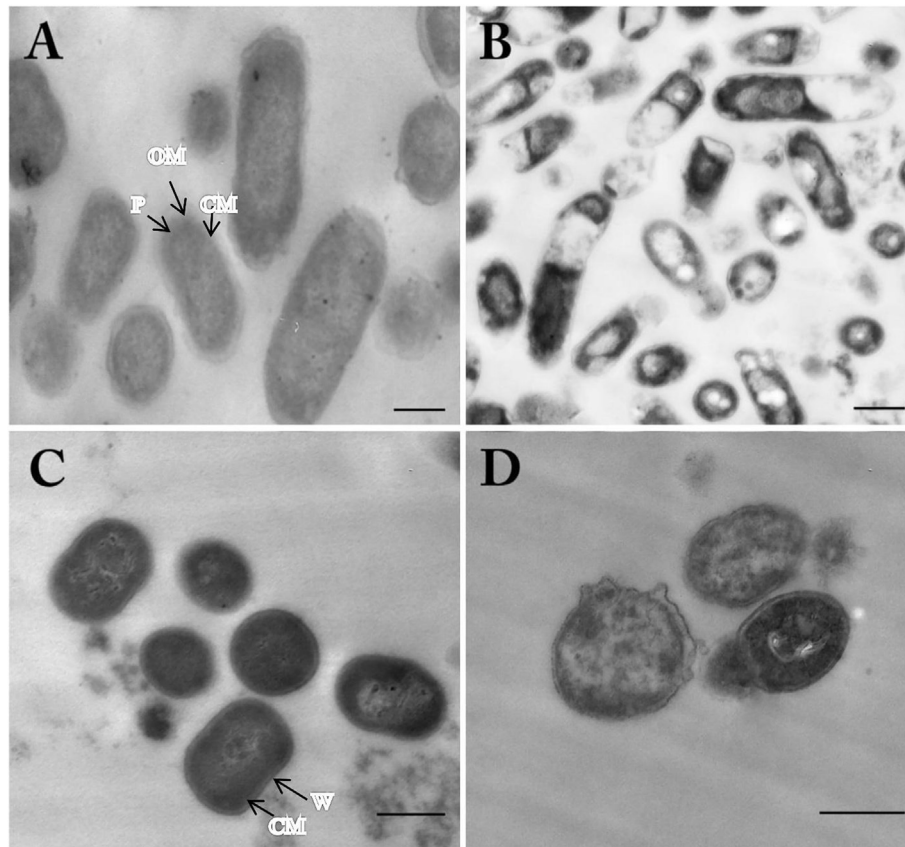


Fig. 3. TEM images of PA-09 strain (B) and SA-17 strain (D) treated with methanol extract of *L. shawii* leaves. Control cells (A&C). Arrows indicate the location of the Cytoplasmic Membrane (CM), Outer Membrane (OM), Periplasmic space and Peptidoglycan (P) and Cell Wall (W). PA, *Pseudomonas aeruginosa*; SA, *Staphylococcus aureus*.

S. aureus 17 (SA-17) was more sensitive to the *L. shawii* extracts than the Gram-negative bacterial strain, *P. aeruginosa* 9 (PA-09) with MIC values ranged between 2 and 16 $\mu\text{g mL}^{-1}$ for the PDR SA-17 strain and with MIC values ranged between 4 and 32 $\mu\text{g mL}^{-1}$ for the MDR PA-09 strain. Additionally, the methanol extract exerted its significant antibacterial activity against both PDR SA-17 and MDR PA-09 strains with MIC values of 2 and 4 $\mu\text{g mL}^{-1}$, respectively (Table 5). The antibacterial activity of the *L. shawii* methanol extract observed in this study was found to be stronger than other methanol extracts.⁴¹ The difference in antibacterial activities of *L. shawii* extracts against the SA-17 and PA-09 strains is more probably due to the ultrastructural differences between Gram-positive and Gram-negative bacteria. The higher resistance of Gram-negative bacteria to a plant extract could be due to the more assembled structure of the semi-permeable outer phospholipid membrane of these bacteria compared with the single mucopeptide layer of the Gram-positive bacterial cell wall.^{9,10} This hypothesis was confirmed further in this study by electron microscopy analysis.

On the other hand, the *in vitro* antifungal activity of the *L. shawii* extracts and their MIC values against two drug-resistant fungal strains, *C. albicans* 11 (CA-11) and *A. niger* 5 (AN-05), are presented in Table 5. The methanol extract showed impressive activity against both fungal strains tested overall other extracts with MIC values of 4 and 8 $\mu\text{g mL}^{-1}$ for CA-11 and AN-05 strains, respectively. In agreement with previous reports,⁴² methanol extracts of other plant leaves demonstrated strong antifungal activity against a number of unicellular and filamentous fungi including *C. albicans*, *A. niger*, *A. flavus*. In general, *L. shawii* alcohol extracts had strong antimicrobial activity than aqueous extraction (Table 5) owing to that alcohol provides a complete extraction of bioactive

compounds with a variety of polarities, while aqueous extracts may not contain some of the less polar compounds.⁴³ It appears that antimicrobial activities are closely correlated to the presence of phenolics and flavonoids with antioxidant activity. The antimicrobial activity of phenolics and flavonoids appears to be related to the hydroxyl groups and the degree of hydroxylation.⁴⁴

Overall, our results indicate that the methanol extract of *L. shawii* leaves is the most promising extract showed the presence of phenolics, flavonoids, alkaloids, tannins, glycosides, terpenoids, steroids and with potential antioxidant, antibacterial and anti-fungal activities. To the best of our knowledge, there are no reports on the antimicrobial potential of *L. shawii* leaf extracts on MDR/PDR *P. aeruginosa*, *S. aureus*, *C. albicans* or *A. niger* growth and ultra-structure. Therefore, to explore the possible mechanism of antimicrobial action of the *L. shawii* methanol extract with the bacterial and fungal cell wall, cell membrane or other intracellular structures, the strains of SA-17, PA-09, CA-11, and AN-05 were subjected to TEM.

3.5. Mechanisms of antimicrobial action

The possible mechanisms of action of *L. shawii* methanol extract on the four drug-resistant strains (SA-17, PA-09, CA-11 and AN-05) were investigated by TEM examination in order to provide valuable insight into this plant species development as a novel antimicrobial agent. TEM images of the bacterial and fungal strains tested are given in Fig. 3&4. DMSO-treated (control) cells of bacterial and fungal strains were intact and confirmed that the DMSO had no injury effect on these control cells (Fig. 3A&C and Fig. 4A&C). Similarly, control cells incubated without DMSO (data not shown)

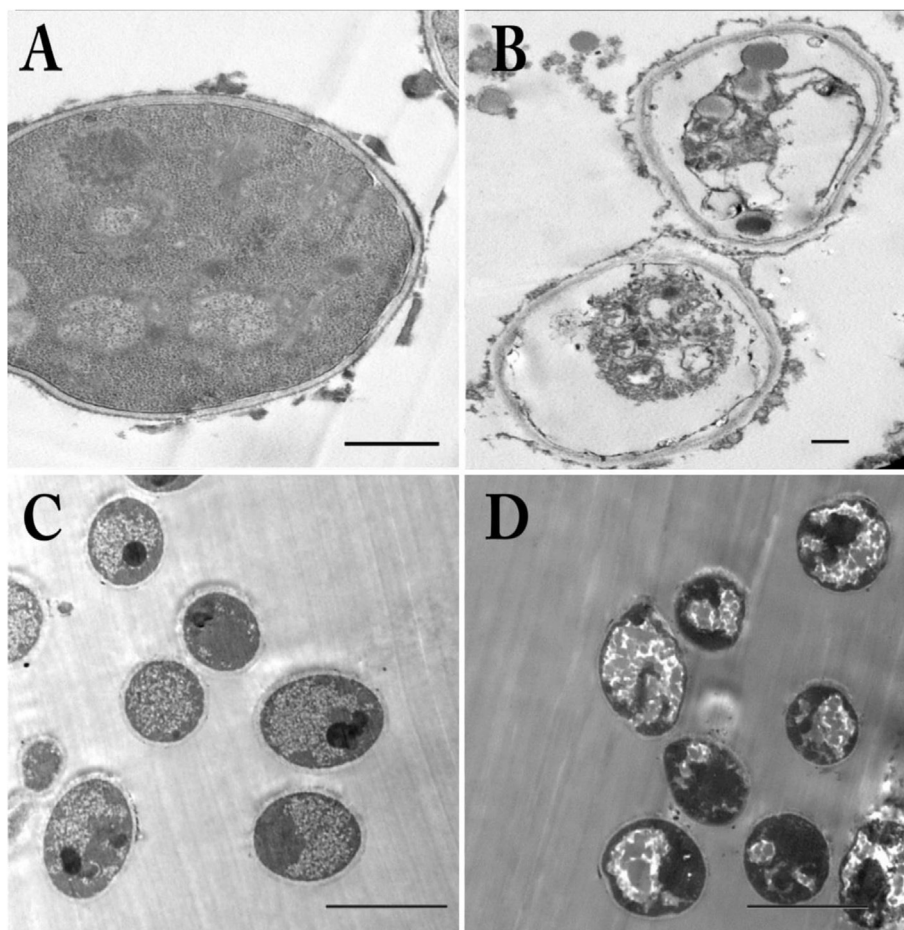


Fig. 4. TEM images of AN-05 strain (B) and CA-11 strain (D) treated with methanol extract of *L. shawii* leaves. Control cells (A&C). AN, *Aspergillus niger*; CA, *Candida albicans*.

confirmed that there were no differences in their ultrastructure compared with DMSO-treated control cells.

Taking into consideration differences in the cell envelope ultrastructure that exist between Gram-negative and Gram-positive bacteria together with the possibility of the *L. shawii* extract that might affect these differences, PA-09 and SA-17 strains as representative species of Gram-negative and Gram-positive bacteria respectively were examined using TEM. The control cells showed a dense cytoplasmic homogeneity (Fig. 3A&C). The cell envelope of PA-09 and SA-17 strains appeared normal and all three layers (outer membrane, periplasmic space, and cytoplasmic membrane) of PA-09 cells (Fig. 3A), as well as the two layers (cell wall and cytoplasmic membrane) of SA-17 cells (Fig. 3C), were discernible. In *L. shawii*-treated PA-09, cells showed partial to complete loss of cytoplasmic electron density, and some cells showed leakage of intracellular cytoplasmic contents (Fig. 3B). Additionally, the cell wall layers of this Gram-negative bacterial strain became separated. This represents further evidence that *L. shawii* disrupts peptidoglycan synthesis. In fact, the outer membrane and peptidoglycan layer of the Gram-negative cell wall are firmly linked by Braun's lipoprotein¹⁰. This lipoprotein requires a properly formed peptidoglycan layer on which to anchor itself. Notably, the Gram-negative cell wall contains a peptidoglycan monolayer, so disruption of its synthesis could result in osmotic cell lysis. On the other hand, the cells of *L. shawii*-treated SA-17 had malformed cell walls, their cytoplasm with less electron dense, the cell wall was thinner than that of control cells, the cytoplasmic membrane was compromised as well as cytoplasmic material from the lysed cells was observed (Fig. 3D).

Our findings strongly suggest that the *L. shawii* extract disrupts cell wall synthesis since the shape of Gram-positive bacteria is governed by the peptidoglycan architecture. In addition, the cell wall mechanical strength is lost and the SA-17 cells undergo osmotic lysis. The observation of an uneven distribution of peptidoglycan (Fig. 3D) suggests that the *L. shawii* leaf extract may be interfering with the membrane proteins, EzrA and GpsB. These proteins play a major coordination role in the formation of peptidoglycan. Clearly, EzrA recruits Penicillin-Binding Proteins (PBPs) to the site of bacterial cell division, and GpsB directs PBPs to the cylindrical part of the Gram-positive bacterial cell following cell division.^{9,10} In conclusion, the methanol extract of *L. shawii* exerts its antibacterial effect as confirmed by ultrastructural changes of the Gram-positive and Gram-negative bacteria strains tested.

Ultrastructural alterations in fungal cells were also examined. In untreated *A. niger* (AN-05) cells, organelles such as nuclei, mitochondria and nucleus are appeared to be normal (Fig. 4A). *L. shawii*-treated AN-05 cells exhibited several changes including stretching of the cell membrane, expansion of endoplasmic reticulum, and leakage of the cell wall, cell membrane and cytoplasmic content (Fig. 4B). The treated *C. albicans* (CA-11) cells showed notable alterations in the morphology including cell wall and cell membrane disintegration (Fig. 4D), while the control CA-11 cells exhibited the typical morphology of *Candida* with uniform central density and endomembrane system enveloped by a regular, typically structured nucleus and intact cell wall (Fig. 4C).

The findings of ultrastructural changes suggest the potential of *L. shawii* leaves as a novel antimicrobial active agent to be exploited

Table 6
Phytochemical compounds, retention time (RT), and biological uses of the *L. shawii* methanol extract using GC-MS.

ID	RT (min)	Peak area (%)	Compound name	Molecular formula	Molecular weight	Compound nature	Uses	References
1	6.203	32.49	P-coumaric acid (4-hydroxycinnamic acid)	C ₉ H ₈ O ₃	164.16	Phenolic	Antimicrobial, antioxidant, anti-inflammatory, analgesic, antiviral, anti-cancer, anxiolytic, antiplatelet aggregation, antipyretic, anti-diabetic and anti-arthritis activities	45
2	6.261	20.18	Apigenin (4,5,6-trihydroxy flavone)	C ₁₅ H ₁₀ O ₅	270.24	Flavonoid	Antioxidant, anti-inflammatory and anti-carcinogenic effects	46
3	6.460	0.474	Hexane (n-hexane)	C ₆ H ₁₄	86.178	Alkane	Food preservative, degreaser, denaturing agent in some alcoholic preparation, perfume preparation, pharmaceutical industry and wound dressing	47
4	9.481	5.73	Catechol (1,2-dihydroxy benzene)	C ₆ H ₆ O ₂	110.1	Phenolic	Antioxidant, synthesis of dyes, rubber, plastics, pharmaceuticals and cosmetics	48
5	12.115	5.37	Hexanol (1-hexanol)	C ₆ H ₁₄ O	102.174	Alcoholic	Perfume preparation	49
6	14.047	5.38	Oxalic acid isobutyl pentyl ester (2-methylpropyl pentyl ethanedioate)	C ₁₁ H ₂₀ O ₄	216.277	Ester	Antimicrobial	50
7	16.081	5.72	Di-n-octyl phthalate (dioctyl 1,2 benzenedicarboxylate)	C ₂₄ H ₃₈ O ₄	390.564	Ester	Anti-cancer, plastic industry, pesticides and cosmetics	51
8	22.911	2.56	Phthalic acid butyl undecyl ester (phthalic acid 1- butyl 2-undecyl ester)	C ₂₃ H ₃₆ O ₄	376.537	Ester	Antimicrobial	52
9	22.222	2.58	Pantolactone (DL-pantoyl lactone)	C ₆ H ₁₀ O ₃	130.14	Oxolanes	Cosmetics	50
10	33.101	19.4	Fisetin (5-desoxyquercetin)	C ₁₅ H ₁₀ O ₆	286.236	Flavonoid	Anti-carcinogenic, antioxidant and anti-inflammatory	53

in controlling wound burn infections caused by drug-resistant *P. aeruginosa*, *S. aureus*, *A. niger* and *C. albicans*. According to our results, the leaves of *L. shawii* are confirmed to have antibacterial, antifungal and antioxidant activities. However, no information is available on biological activities of chemical constituents of this plant species leaves. Henceforth, GC-MS analysis was investigated to explore the phytochemical compounds of *L. shawii* methanol leaf extract.

3.6. GC-MS analysis

GC-MS has become one of the most important techniques for the separation of phytoconstituents as well as for identification of plant secondary metabolites. The results of the GC-MS analysis revealed the identity of 10 compounds from the methanol extract of *L. shawii* leaves (Table 6; Supplemental information, Fig. S2&S3). These compounds are known to possess several biological activities and industrial applications such as antimicrobial, antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, food preservative, degreaser, perfume preparation, wound dressing, pharmaceuticals, cosmetics and anti-arthritis activities (Table 6). The GC and MS

represent the relative concentration and the identification of various compounds of *L. shawii* eluted at a different retention time, respectively. P-coumaric acid (phenolic compound), apigenin (flavonoid compound) and fisetin (flavonoid compound) are the major identified three compounds representing 32.49, 20.18 and 19.4%, respectively. Isolation of P-coumaric acid from *L. shawii* extract was achieved by thin layer chromatography followed by HPLC which validated by optimized chromatographic conditions (data not shown). Chromatogram (Fig. 5) showed P-coumaric acid, which confirmed by a peak at retention time of 6.750 min. To the best of our knowledge, this is the first report to analyze the phytochemical composition of *L. shawii* leaves. Plants are the promising natural source for discovering novel compounds with medicinal importance for drug development. Overall, *L. shawii* leaves provide a clear scientific basis that this plant species can be a potent source for the novel medicines. Accordingly, our results encourage further studies on *L. shawii* leaves and identifying particular active phytochemical constituents responsible for the specific biological activity in order to standardize the plant preparation for maximum therapeutic application against burn wounds and other infections related to drug-resistant clinical pathogens.

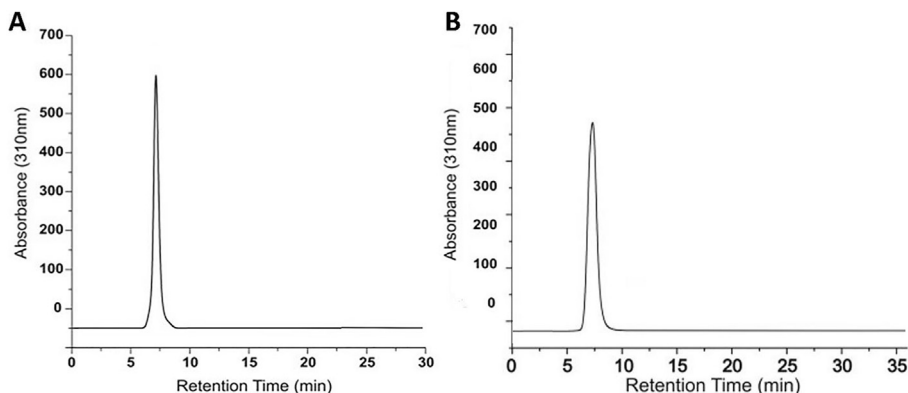


Fig. 5. Chromatogram of P-coumaric acid standard (A) and that of the *L. shawii* methanol extract (B).

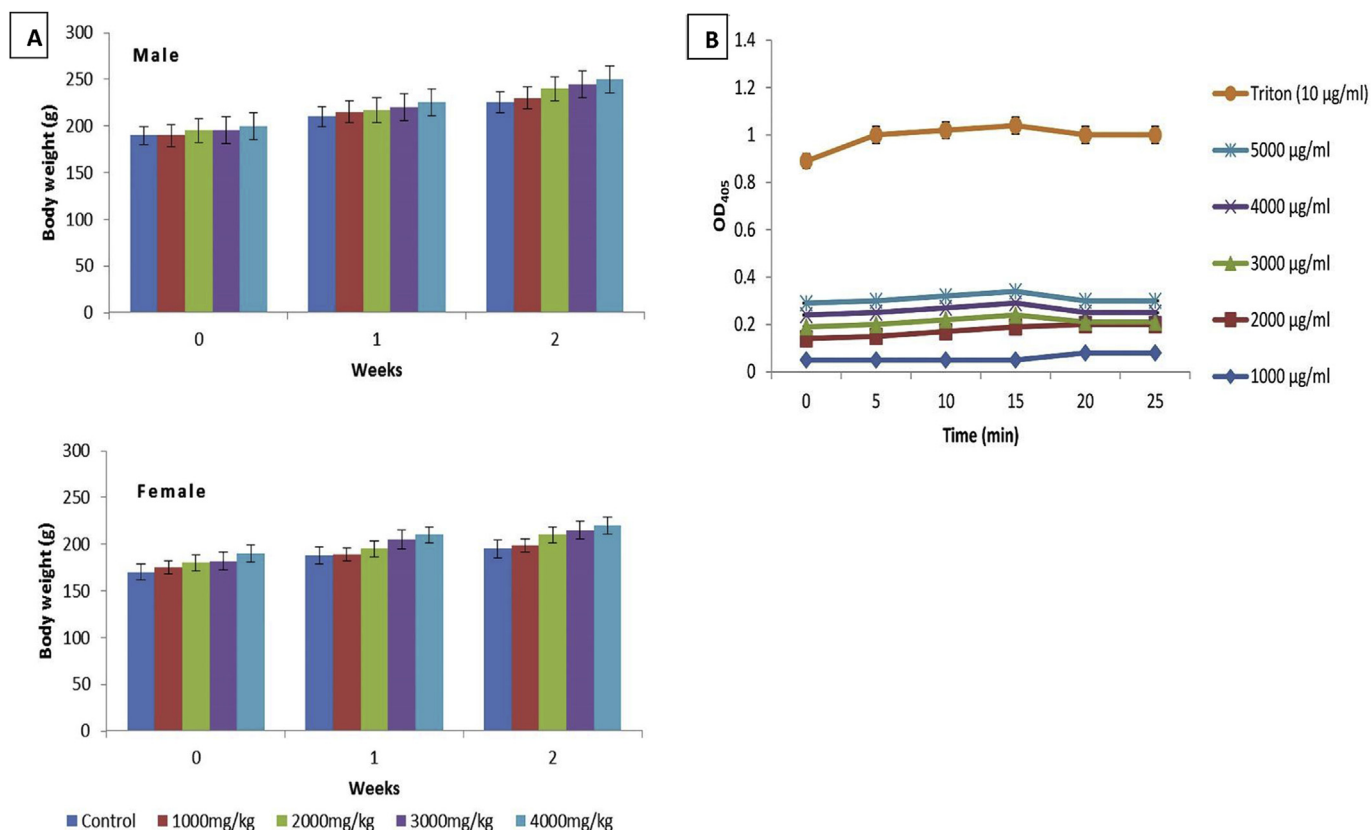


Fig. 6. *In vivo* toxicity assessment of methanol extract from *L. shawii* leaves in male and female groups of albino rats after acute oral administration; average weight of rats in the treated groups were compared with that of the respective control group ($P \leq 0.05$) (A). Effect of methanol extract of *L. shawii* leaves on haemolytic activities; Triton X-100 is a positive control (B).

3.7. Toxicological assessment

To the best of our knowledge, the toxicological evaluation of the methanol extract from *L. shawii* leaves has not been conducted. In order to confirm the safety of phytochemical constituents in humans, toxicological studies in experimental animals were performed to assess the potential toxicity and thus to provide a direction for determining a dose regimen for human use. Fig. 6A shows the assessment of methanol extract from *L. shawii* leaves in albino rats after acute oral administration. Oral dose administration of the methanol extract up to the highest concentration of 4000 mg/kg body weight revealed no clinical signs and did not cause any mortality. For both male and female rats, no significant difference in the body weight was observed between *L. shawii*-treated groups and the control group at the end of experiment periods. According to these results the acute lethal dose (LD_{50}), expressed as weight of methanol extract tested per unit weight of albino rat (mg/kg), was assumed to be greater than 4000 mg/kg suggesting that this plant leaf extract could be generally regarded as nontoxic depending on the scale of Hodge and Sterner.⁵⁴ Body weight is often the most sensible indicator of an adverse effect.⁵⁵ The loss of body weight in a period of 7 days is considered as the indicator that provides more information in toxicological assessment. In addition, the increase in body weight for treated albino rat groups was considered normal (Fig. 6A).

The haemolytic activity assay has proven to be an alternative method for simple cytotoxicity assessment. As presented in Fig. 6B, no significant haemolytic activity of albino rats' red blood cells treated with *L. shawii* extract at concentrations of 1000–5000 $\mu\text{g mL}^{-1}$ was observed compared to the positive control (Triton X-100), which

supports the safety of this leaf extract. The toxicological evaluation of different plants using *in vitro* haemolytic activity assay has been reported in other studies.^{56,57} Collectively, toxicological assessment using rat model supports the safety of this plant species for oral use. However, so far, there is no available toxicological data on human regarding the *L. shawii* leaf extracts, therefore further assessment should be performed to define precisely the safety doses of this novel leaf extract for human use.

4. Conclusions

In the last decade, an inevitably increases in clinical bacterial and fungal pathogens conferring MDR or PDR to various antibacterial and antifungal agents has been witnessed. Therefore, there is a direct need to discover novel plant-based biological macromolecules as alternative natural antimicrobial agents with diverse chemical structures and novel mechanisms of action. Our findings clearly revealed that all the tested extracts exhibited significant total phenolics and flavonoids and displayed a significant correlation between the antioxidant activities and total phenolic and flavonoid compounds implied that these are the major contributors of antioxidant activities of *L. shawii* leaves. Additionally, *L. shawii* extracts contained the considerable potential of antimicrobial activities against MDR/PDR *P. aeruginosa*, *S. aureus*, *C. albicans* and *A. niger* clinical strains. The GC-MS analysis identified potential bioactive and functional compounds. Of those, P-coumaric acid, apigenin and fisetin are the major identified compounds. The *in vivo* toxicological assessment of methanol extract obtained from leaves of *L. shawii* showed neither any clinical signs of acute toxicity nor any mortality, suggesting that this plant leaf extract could be

generally regarded as nontoxic. No significant haemolytic activity of *L. shawii* methanol extract against erythrocytes was found which indicated that *L. shawii* leaves might be safe for the use of pharmaceutical and natural therapies. Considering these results, *L. shawii* leaves would certainly pave the path for the development of novel antimicrobial and antioxidant agents that could potentially combat clinical MDR/PDR pathogens.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtcme.2019.01.004>.

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