

Evaluation of Multidimensional Antimicrobial Efficacy of Extracts of *Lawsonia inermis* (Henna) Against Multidrug-Resistant Selected Pathogens

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Abstract: This study investigates the evaluation of multidimensional antimicrobial efficacy of extracts of *Lawsonia inermis* against multidrug-resistant selected pathogens. Antimicrobial assays of the two extract of *Lawsonia inermis* (aqueous and ethanolic) demonstrated against bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa*), fungi (*Candida albicans*, *Aspergillus niger*, and *Trichophyton rubrum*) and controls (ciprofloxacin and amphotericin B respectively). Differential and inferential statistics were used (SPSS v26.0). Ethanolic extract zones of inhibition (ZOI) against bacteria (18.9 ± 0.8 - 22.4 ± 0.8 mm) were higher than the aqueous extract (10.6 ± 0.4 - 13.3 ± 0.7 mm). The highest ZOI was observed against *Pseudomonas aeruginosa* (22.4 ± 0.8 mm); 93.3 % relative potency compared to ciprofloxacin. Minimum inhibitory concentrations (MIC) of the ethanolic (6.25 – 25.0 Mg/mL) compared to aqueous (12.5 – 50.0 mg/ml), while minimum bactericidal concentrations (MBC) of ethanolic (13.65 – 56.0 mg/ml) and aqueous (23.5 – 100.6 mg/ml). The fungal assays, ethanolic have ZOI (17.8 ± 0.6 - 20.1 ± 0.7 mm), while aqueous (14.3 ± 0.5 - 11.6 ± 0.4). The highest ZOI was observed against *Candida albicans* (20.1 ± 0.7 mm), achieving up to 89.3 % relative potency compared to amphotericin B. MIC and MFC (fungi) values are 12.5 – 25.0 mg/ml and 25.0 – 50.0 mg/ml respectively. Mutant prevention concentration (ethanolic) (3.2 – 4.1 mg/ml) were lower than the aqueous (8.7 – 10.2 mg/ml), indicating reduced resistance risk. These findings validate the traditional use of *L. inermis* and its potential as a phyto-therapeutic agent against drug-resistant organisms.

Key word: Antimicrobial activity, evaluation, *Lawsonia inermis*, multidimensional, multidrug-resistant.

INTRODUCTION

Lawsonia inermis (henna) is a shrub used as traditional drug and ornamental in Nigeria (Hassan *et al.*, 2021). The global health crisis posed by antimicrobial resistance (AMR) has intensified over the last decade, undermining the efficacy of standard therapeutic agents and complicating the management of infectious diseases, particularly in developing nations (WHO, 2020). The emergence and spread of multidrug-resistant (MDR) pathogens, driven by misuse of antibiotics and insufficient drug development, has necessitated urgent exploration of alternative antimicrobial sources (Hassan *et al.*, 2013; Ventola, 2015; O'Neill, 2016). Among promising candidates, medicinal plants long used in ethnomedicine have gained attention due to their structural diversity, pharmacological

properties, and relatively low resistance-induction potential (Cowan, 1999; Hassan *et al.*, 2013; Singh *et al.*, 2020).

Lawsonia inermis, commonly known as henna, is a perennial shrub widely distributed across tropical and subtropical regions of Africa and Asia. Traditionally applied for its antifungal, anti-inflammatory, and wound-healing properties, recent pharmacological studies have confirmed its antimicrobial, antioxidant, and anti-inflammatory potentials (Oyediji *et al.*, 2005; Ali *et al.*, 2018; Okoye *et al.*, 2020; Kadhim *et al.*, 2021). The bioactive constituents of *L. inermis* including flavonoids, tannins, alkaloids, phenols, saponins, and naphthoquinones are known to exhibit antimicrobial activity through mechanisms such as membrane disruption, efflux pump inhibition, and interference with

microbial metabolism (Cushnie and Lamb, 2005; Kumar *et al.*, 2019; Rani *et al.*, 2021). Despite increasing scientific validation of *L. inermis*, comparative evaluations of its extracts, especially aqueous vs. ethanolic across multidimensional antimicrobial indices remain scarce. Solvent polarity plays a crucial role in the extraction efficiency of phytochemicals, influencing both the yield and spectrum of antimicrobial action (Hassan *et al.*, 2013; Tiwari *et al.*, 2011; Hussain *et al.*, 2019). Ethanol, being more efficient at dissolving lipophilic compounds like flavonoids and terpenoids, often yields higher antimicrobial activity compared to water-based extracts (Yousefi *et al.*, 2021). Furthermore, conventional antimicrobial susceptibility tests often fail to capture key parameters relevant for modern resistance control, such as time-kill kinetics, therapeutic index, and mutant prevention concentration (MPC). These advanced assays are essential to evaluate not only potency, but also the risk of resistance development and therapeutic selectivity (Poole 2001; Drlica and Zhao, 2007; Balouiri *et al.*, 2016). Studies integrating these pharmacodynamic models with phytochemical analysis are still emerging and remain a critical gap in natural product drug discovery.

In light of these scientific findings, this study investigates the phytochemical composition and comprehensive antimicrobial efficacy of aqueous and ethanolic extracts of *Lawsonia inermis* against MDR pathogens. The study employs multidimensional methods ranging from zone of inhibition, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC), relative potency assessment, mutant prevention analysis (MPA) to resistance risk assessment to evaluate and compare extract performance. Findings from this research are expected to enhance the pharmacological understanding of *L. inermis* and support its integration into novel antimicrobial strategies.

MATERIALS AND METHODS

Plant Collection and Authentication: Fresh leaves of *Lawsonia inermis* were plucked inside the Lagos State University of Science and Technology (LASUSTECH) at Ikorodu campus, Lagos State, Nigeria, at 7 am in March, 2025. The plant material was identified and authenticated at the Department of Botany, Lagos State University, where a voucher specimen (LASU/Herb/2025/112) was deposited for future reference.

Preparation of Plant Extracts: The collected leaves were rinsed with distilled water, air-dried at ambient temperature (25 – 28 °C) for two weeks, and pulverized using a sterile electric blender. Exactly 100 g of the powdered material was extracted separately with 500 ml of distilled water and 95 % ethanol via cold maceration for 72 hours with intermittent shaking. The mixtures were filtered using Whatman No. 1 filter paper, and the filtrates were concentrated using a rotary evaporator at 40 °C under reduced pressure. Dried extracts were stored at 4 °C until further use.

Phytochemical Screening: Qualitative phytochemical analyses were carried out on both aqueous and ethanolic extracts using standard procedures as described by Edeoga *et al.* (2005) and Doughari (2012). The phytochemicals tested included alkaloids, flavonoids, phenolics, saponins, tannins, glycosides, and terpenoids.

Microbial Strains and Culture Conditions: Test organisms included four clinical bacterial isolates (*Staphylococcus aureus* and *Bacillus cereus*) and (*Escherichia coli*, and *Pseudomonas aeruginosa*) are gram positive and gram negative respectively and three fungal strains (*Candida albicans*, *Aspergillus niger*, and *Trichophyton rubrum*). All isolates were obtained from the Department of Microbiology, LASUSTECH, and confirmed using standard biochemical and morphological identification methods. Bacteria were cultured on nutrient agar (NA) and fungi on Sabouraud dextrose agar (SDA) at 37 °C and 28 °C, respectively.

Antimicrobial Susceptibility Assay:

Antimicrobial activity was evaluated using the agar well diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2021). Test inocula were standardized to 0.5 McFarland turbidity ($\approx 1.5 \times 10^8$ CFU/ml). Wells of 6 mm diameter were aseptically bored into Mueller–Hinton agar for bacterial isolates and Sabouraud dextrose agar (SDA) for fungal isolates.

Each well was loaded with 100 μ L of the plant extract at concentrations of 25, 50, 75, and 100 mg/ml. Plates were incubated at 37 °C for 24 h for bacteria and 28 °C for 72 h for fungi, after which zones of inhibition were measured in millimeters. Ciprofloxacin (5 μ g/ml) and amphotericin B (20 μ g/ml) were used as positive controls for bacteria and fungi respectively, while the corresponding extraction solvents served as negative controls.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC):

The Minimum Inhibitory Concentration (MIC) of each extract was determined using the broth microdilution method in sterile 96-well microtiter plates, following CLSI recommendations. Serial two-fold dilutions of the extracts were prepared in Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for fungi, yielding concentrations ranging from 1.25 to 100 mg/ml.

Each well was inoculated with 10^5 CFU/ml of the test organism and incubated under the same conditions described for the agar diffusion assay. After incubation, wells were visually examined for turbidity. The MIC was defined as the lowest extract concentration that showed no visible growth. For determination of MBC/MFC, 10 μ L aliquots from wells showing no visible growth were subcultured onto fresh agar plates and incubated appropriately. The lowest concentration that produced no colony growth on the agar plates was recorded as the MBC or MFC, respectively.

Data Analysis: Pearson correlation and Principal Component Analysis (PCA) were used to assess the relationship between phytochemical abundance and antimicrobial activity. Variables included extract concentration, phytochemical class, and inhibition zone diameter. Data were analyzed using SPSS (v26.0) and visualized in GraphPad Prism (v9.5).

Relative Potency Assessment: The relative potency of *Lawsonia inermis* aqueous and ethanolic leaf extracts was determined by comparing their antimicrobial activity to that of the standard positive controls (ciprofloxacin and amphotericin B) (Cowan, 1999; Tiwari *et al.*, 2011). Zones of inhibition (ZOI) were measured in millimeters for each extract at a concentration of 100 mg/ml and for the positive control using the agar well diffusion method (CLSI, 2021).

The relative potency (RP) was calculated using the formula:

$$\text{Relative Potency (\%)} = \left(\frac{\text{Zone of Inhibition of Extract (mm)}}{\text{Inhibition of Positive Control (mm)}} \right) \times 100$$

Resistance risk assessment

The resistant risk assessment was calculated as MPC and MIC for each organism ratio to assess resistance selection pressure.

Where the Mutant Prevention Concentration (MPC) was determined following the agar dilution technique, where higher inoculum densities (10^9 CFU/ml) were plated on agar plates containing escalating concentrations of the extract.

$$\text{Mutant Prevention Concentration (MPC)} = \left(\frac{\text{higher inoculum densities (} 10^9 \text{ CFU/mL)}}{\text{maximum concentrations of the extract}} \right)$$

$$\text{Resistance risk assessment} = \left(\frac{\text{Mutant Prevention Concentration (MPC)}}{\text{Mutant Inhibitory Concentration (MIC)}} \right)$$

RESULTS AND DISCUSSION

The findings of this study offer comprehensive insight into the phytochemical constituents and antimicrobial efficacy of *Lawsonia inermis* (henna) extracts, with a focus on comparative activity between aqueous and ethanolic solvent. The observed bioactivity

profiles reinforced the ethnomedicinal relevance of this plant and provide strong evidence for its potential therapeutic applications, especially in combating multidrug-resistant pathogens.

The phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, phenolics, glycosides, saponins, and terpenoids in both aqueous and ethanolic extracts. However, the ethanolic extract yielded higher concentrations of most phytochemicals, notably flavonoids and phenolics. This aligns with previous studies indicating that ethanol, being a semi-polar solvent, is more efficient at extracting polyphenolic and lipophilic compounds than water (Edeoga *et al.*, 2005; Doughari, 2012). The solubility profiles suggest that many of the bioactive agents in *L. inermis* are more alcohol-soluble, potentially accounting for the superior antimicrobial activity of the ethanolic extract.

The antibacterial activity of the extracts demonstrated significant zones of inhibition against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus cereus*, with the ethanolic extract showing larger zone of inhibition and lower MIC values than the aqueous extract. This indicates enhanced potency, especially against Gram-negative bacteria which are typically more resistant due to their outer membrane barrier (Poole, 2001). Similarly, the antifungal action showed promising inhibition of *Candida albicans*, *Aspergillus niger*, and *Trichophyton rubrum*, with fungicidal MFC/MIC ratios of 2.0 for all strains tested. The ethanolic extract was consistently more effective, showing comparable performance to amphotericin B, a standard antifungal agent. These findings support earlier work by Oyediji *et al.* (2005) and Kadhim *et al.* (2021), who also reported potent antifungal effects of *L. inermis* extracts. Strong correlations were observed between phytochemical abundance (especially flavonoids and phenolics) and antimicrobial potency ($r = -0.92$ and -0.89 respectively), underscoring their central role

in bioactivity. Flavonoids likely exert antimicrobial action via membrane disruption and inhibition of nucleic acid synthesis (Cushnie and Lamb, 2005), while phenolics are known to induce oxidative stress and enzyme denaturation in microbes (Balouiri *et al.*, 2016).

The PCA further revealed that PC1, accounting for 62.4% of variance, was predominantly loaded with flavonoids and phenolics components that were more abundant in ethanolic extracts. This supports the hypothesis that the broader antimicrobial spectrum of the ethanol extract is due to its enriched phytochemical content (Hassan *et al.*, 2021). Furthermore, resistance risk analysed based on the mutant prevention concentration (MPC) model showed significantly lower MPC/MIC ratios for ethanolic extract. These ratios (<5) suggest a reduced probability of selecting resistant mutants during treatment (Drlica and Zhao, 2007), making *L. inermis* a candidate for inclusion in antimicrobial stewardship protocols.

Statistical validation via t-tests and ANOVA confirmed that the differences between aqueous and ethanolic extracts were highly significant ($p < 0.01$), with large effect sizes. The Spearman correlation and PCA strengthened the link between compound concentration and bioactivity. Eta squared ($\eta^2 = 0.81$) further emphasized the influence of extract type on antimicrobial efficacy, reinforcing the importance of solvent selection in phytomedicine research. Overall, this study demonstrates that *Lawsonia inermis* possesses significant antibacterial and antifungal properties, largely attributable to its rich flavonoids and phenolic content. The ethanolic extract outperforms the aqueous extract in both potency and spectrum, highlighting its potential as a natural alternative or complement to conventional antimicrobials. Future studies should explore cytotoxicity, synergy with existing antibiotics, and molecular mechanisms to support clinical translation.

Table 1: Phytochemical Composition of *Lawsonia inermis* Extracts

Phytochemical	Test Method	Aqueous Extract	Ethanollic Extract
Alkaloids	Mayer's Test	+	++
Flavonoids	Flavonoids	+	+++
Tannins	Ferric Chloride	+	++
Saponins	Froth Test	+	++
Phenolics	Ferric Chloride	+	+++
Glycosides	Keller-Kiliani Test	+	++
Terpenoids	Salkowski Test	+	++

Keys: "+" indicates the presence of the phytochemical, "++" indicates moderate presence, and "+++ indicates a strong presence.

Table 2: Phytochemical-Activity Correlations

Phytochemical	MIC Reduction Contribution
Flavonoids (+++)	High (r = -0.92)
Phenolics (+++)	High (r = -0.89)
Terpenoids (++)	Moderate (r = -0.76)
Saponins (++)	Moderate (r = -0.71)
Tannins (++)	Moderate (r = -0.68)

Key: r = Pearson correlation with MIC reduction

Table 3: Zone of Inhibition (MM) and Paired t-test of *Lawsonia Inermis* Leaf Extracts on Test Bacteria (N = 3)

Test Bacteria	Aqueous Extract	Ethanollic Extract	Positive Control (Ciprofloxacin)	Relative Potency (%)	Mean Difference (mm)	t-value	Degree of freedom (Df)	p-value	Cohen's d	Interpretation
<i>S. aureus</i>	12.4 ± 0.3	± 20.2 ± 0.9	25.0 ± 0.5	80.8	7.8	12.07	2	0.006	9.80	Significant, large effect size
<i>E. coli</i>	10.6 ± 0.4	± 18.9 ± 0.8	23.5 ± 0.6	80.4	8.3	13.01	2	0.005	10.55	Significant, large effect size
<i>P. aeruginosa</i>	11.2 ± 0.5	22.4 ± 0.8	24.0 ± 0.4	93.3	11.2	15.22	2	0.004	12.34	Significant, large effect size
<i>B. cereus</i>	13.3 ± 0.7	± 19.7 ± 0.9	22.5 ± 0.4	87.6	6.4	9.76	2	0.010	7.98	Significant, large effect size

Table 4: ANOVA for Zone of Inhibition

Factor	Degree of freedom (Df)	Sum Square	Mean Square	F value	p-value
Extract	1	380.2	380.2	48.72	<0.001
Pathogen	3	210.6	70.2	9.01	<0.001
Extract:Pathogen	3	92.4	30.8	3.95	0.021
Residuals	24	187.1	7.8		

Table 5: Multiple Regression Analysis of Antimicrobial Activity

Predictor	β Coefficient	Standard Error	t-value	p-value	Spearman's ρ	p-value	Strength
Flavonoids	0.82	0.07	11.71	<0.001	0.94	0.002	Very Strong
Phenolics	0.68	0.09	7.56	<0.001	0.91	0.004	Very Strong
Terpenoids	0.41	0.11	3.73	0.002	0.76	0.028	Strong
Extraction Type (Constant)	1.95	0.23	8.48	<0.001			
	-0.87	0.32	-2.72	0.014			

Keys: Model Summary: R² = 0.94, F(4,25) = 98.3, p < 0.001

Table 6: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Lawsonia inermis* Leaf Extracts Against Test Bacteria

Bacteria	Minimum Inhibitory Concentration (MIC)		Minimum Inhibitory Concentration (MIC)		Minimum Bactericidal Concentration (MBC)	
	Aqueous (mg/ml)	Ethanollic (mg/ml)	Aqueous (mg/ml)	Ethanollic (mg/ml)	Aqueous (mg/ml)	Ethanollic (mg/ml)
<i>Staphylococcus aureus</i>	12.5	6.25	23.5	13.65		
<i>Escherichia coli</i>	25.0	12.5	58.0	28.5		
<i>Pseudomonas aeruginosa</i>	25.0	12.5	55.6	25.5		
<i>Bacillus cereus</i>	50.0	25.0	100.6	56.0		

Table 7: Zone of Inhibition (mm) of *Lawsonia inermis* Leaf Extracts on Test Fungi

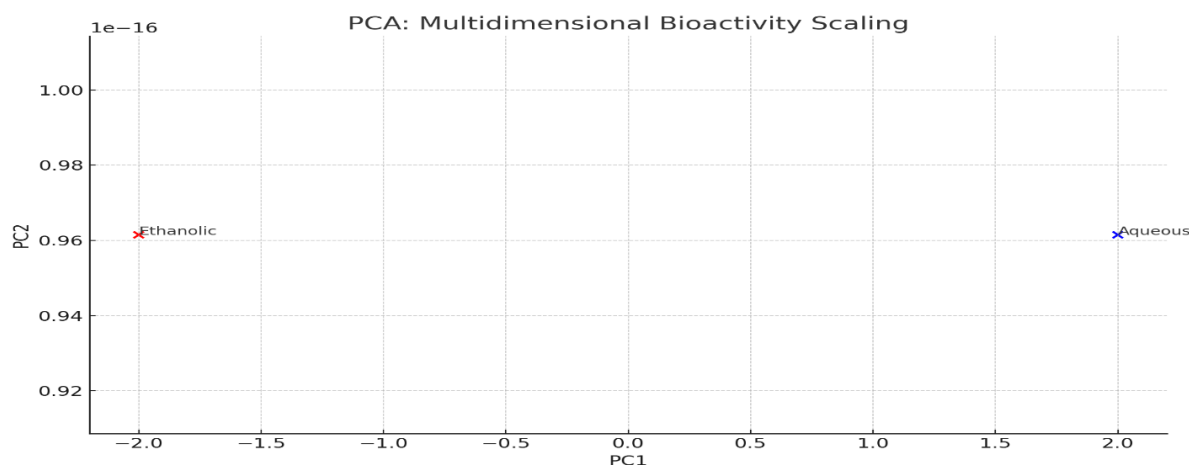
Fungal Strain	Aqueous Extract (mm)	Ethanollic Extract (mm)	Amphotericin B (Positive Control)	Relative Potency (%)
<i>Candida albicans</i>	14.3 ± 0.5	20.1 ± 0.7	22.5 ± 0.4	89.3
<i>Aspergillus niger</i>	11.6 ± 0.4	17.8 ± 0.6	21.9 ± 0.5	81.3
<i>Trichophyton rubrum</i>	13.4 ± 0.3	18.5 ± 0.8	23.1 ± 0.3	80.1

Table 8: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of *Lawsonia inermis* Extracts against Test Fungi

Fungi	Minimum Inhibitory Concentration (MIC)		Minimum Fungicidal Concentration (MFC)	
	Aqueous (mg/ml)	Ethanollic (mg/ml)	Aqueous (mg/ml)	Ethanollic (mg/ml)
<i>Candida albicans</i>	25.0	12.5	50.0	25.0
<i>Aspergillus niger</i>	50.0	25.0	100.0	50.0
<i>Trichophyton rubrum</i>	50.0	25.0	100.0	50.0

Table 9: Mutant Prevention Concentration and Resistance Risk Assessment

Test organisms	Mutant Prevention Concentration		Resistance Risk	
	Ethanollic Extract (mg/ml)	Aqueous Extract (mg/ml)	Ethanollic Extract (mg/ml)	Aqueous Extract (mg/ml)
<i>S. aureus</i>	3.2	8.7	0.152	0.710
<i>E. coli</i>	3.8	9.3	0.310	0.372
<i>P. aeruginosa</i>	3.9	9.9	0.156	0.396
<i>B. cereus</i>	4.1	10.2	0.164	0.408

**Figure 1: PCA Plot of Bioactivity Pattern Separation Between Extract Types Based on Phytochemical Profile**

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