RESEARCH PAPER

Antimicrobial Activity of a Standardized Polyherbal Formulation Comprising *Phyllanthus urinaria* L., *Murraya koenigi*i (L.) *Spreng.*, and *Delonix regia* (Boj. ex Hook.) Raf.: Phytochemical Profiling, Acute Toxicity, and Broad-Spectrum Bactericidal/Fungicidal Efficacy

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ABSTRACT

Background: Antimicrobial resistance (AMR) is among the foremost threats to global public health, and the shrinking pipeline of genuinely new antibiotic classes has renewed interest in multi-component botanical therapeutics. Polyherbal formulations (PHFs), a cornerstone of traditional medicine, are proposed to achieve antimicrobial synergy through multi-target phytochemical action, potentially lowering the probability of resistance development relative to single-target synthetic agents.

Objective: To prepare, phytochemically standardize, and evaluate the acute safety and antimicrobial potential of a polyherbal formulation combining Phyllanthus urinaria L., Murraya koenigii (L.) Spreng., and Delonix regia (Boj. ex Hook.) Raf.

Methods: Equal proportions of the three dried, powdered plant materials were subjected to successive solvent extraction (petroleum ether, ethyl acetate, acetone, ethanol, and hydroalcoholic solvent). Extracts were characterized by qualitative phytochemical screening and thin-layer chromatographic (TLC) fingerprinting. Acute oral toxicity was assessed in adult female Wistar rats according to OECD Guideline 420. Antimicrobial activity of the polyherbal formulation was evaluated by agar well diffusion, broth micro-dilution (minimum inhibitory concentration, MIC), and determination of the minimum bactericidal/fungicidal concentration (MBC/MFC) against three Gram-positive bacteria, three Gram-negative bacteria, and two fungal strains, using ciprofloxacin and ketoconazole as reference standards.

Results: The hydroalcoholic (14.10 ± 0.31%) and ethanol (11.50 ± 0.25%) extracts gave the highest yields and the broadest phytochemical profiles, dominated by flavonoids, tannins, and phenolics. No mortality or overt toxicity occurred at 2000 mg/kg body weight, establishing an oral LD_{50} > 2000 mg/kg. The formulation produced concentration-dependent zones of inhibition against all eight test organisms, with MIC values ranging from 62.5 to 250 µg/mL and MBC/MFC-to-MIC ratios of 2.0 to 4.0, confirming bactericidal/fungicidal rather than merely static action against every organism tested. Bacillus subtilis was the most susceptible organism (MIC 62.5 µg/mL), and Pseudomonas aeruginosa the least susceptible (MIC 250 µg/mL); mean antimicrobial activity relative to the reference standards, calculated across all eight organisms at 500 µg/mL, was 70.2%.

Conclusion: The polyherbal formulation exhibits broad-spectrum, concentration-dependent, and predominantly cidal antimicrobial activity against clinically relevant Gram-positive, Gram-negative, and fungal pathogens, combined with a wide margin of acute oral safety. These findings support its further development as a standardized botanical antimicrobial candidate, pending bioassay-guided fractionation, mechanistic, and clinical studies.

Keywords: Polyherbal formulation; Phyllanthus urinaria; Murraya koenigii; Delonix regia; antimicrobial activity; antimicrobial resistance; minimum inhibitory concentration; phytochemical screening; acute oral toxicity

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1. Introduction

Antimicrobial resistance (AMR) has emerged as one of the most serious challenges facing modern medicine. The World Health Organization has ranked AMR among the ten leading global public health threats confronting humanity (World Health Organization [WHO], 2021), and a landmark 2022 analysis published in The Lancet attributed 1.27 million deaths directly to bacterial AMR in 2019, with resistant infections associated with a further 4.95 million deaths, making AMR a leading cause of mortality worldwide (Murray et al., 2022). The rapid emergence and dissemination of multidrug-resistant, extensively drug-resistant, and pandrug-resistant pathogens — including methicillin-resistant Staphylococcus aureus, vancomycin-resistant enterococci, extended-spectrum β-lactamase (ESBL)-producing Enterobacterales, carbapenem-resistant Pseudomonas aeruginosa, and azole-resistant Candida species — has steadily eroded the effectiveness of first-line antimicrobial agents, contributing to higher rates of treatment failure, prolonged hospitalization, escalating healthcare costs, and increased mortality (Tacconelli et al., 2018).

Compounding this crisis is a well-documented "discovery void" in antibacterial drug development: the number of genuinely novel antibacterial classes reaching clinical use has slowed markedly over the past four decades even as resistance mechanisms continue to proliferate (Theuretzbacher et al., 2020). Existing antimicrobial agents are further constrained by narrow spectra of activity, organ-specific toxicity (nephrotoxicity, hepatotoxicity, ototoxicity, and Clostridioides difficile-associated diarrhoea), and the high cost of newer broad-spectrum agents, which limits accessibility in resource-constrained settings. These limitations have renewed scientific interest in natural products as a source of structurally novel antimicrobial scaffolds capable of circumventing existing resistance pathways.

Traditional systems of medicine — including Ayurveda, Siddha, Unani, Traditional Chinese Medicine, and regional folk practices — represent an extensive, comparatively underexplored reservoir of such scaffolds. The WHO estimates that approximately 80% of the population in developing countries relies primarily on plant-based remedies for their primary healthcare needs (WHO, 2019), and roughly one-third of small-molecule drugs approved by the United States Food and Drug Administration between 1981 and 2019 were natural products, natural product derivatives, or natural product mimics (Newman & Cragg, 2020). Within traditional medicine, the polyherbal formulation (PHF) — a deliberate combination of multiple herbs in defined proportions — occupies a central place, particularly within Ayurvedic and Siddha practice. The rationale for combining herbs rather than relying on a single-plant extract rests on several pharmacologically plausible mechanisms of synergy: multi-target action against distinct components of a pathogen's physiology, pharmacokinetic synergy in which one constituent enhances the absorption, distribution, or stability of another, and modulation of resistance mechanisms — for example, inhibition of microbial efflux pumps or β-lactamases by one phytoconstituent potentiating the antimicrobial action of another (Williamson, 2001; Wagner & Ulrich-Merzenich, 2009). Because the simultaneous emergence of resistance against several unrelated molecular targets is statistically far less probable than resistance to a single target, multi-component botanical antimicrobials have been proposed to carry an inherently lower risk of driving resistance than single-target synthetic agents (Bueno, 2016), a principle consistent with the broader systems-pharmacology paradigm for multifactorial disease (Hopkins, 2008; Zhou et al., 2016).

The present study evaluates a PHF prepared from three plants with independent histories of traditional antimicrobial use. Phyllanthus urinaria L. (Phyllanthaceae) is a hepatoprotective and antimicrobial herb used across Ayurvedic, Siddha, and Traditional Chinese Medicine traditions, rich in hydrolysable tannins such as corilagin and geraniin, alongside flavonoids including quercetin, kaempferol, and rutin (Calixto et al., 1998; Liu et al., 2014; Mao et al., 2016). Murraya koenigii (L.) Spreng. (Rutaceae), the culinary curry-leaf tree, is characterized by carbazole alkaloids — including mahanimbine, murrayanine, and girinimbine — that possess documented antibacterial activity in addition to their better-studied antidiabetic effects (Ningaiah et al., 2014; Nagappan et al., 2017). Delonix regia (Boj. ex Hook.) Raf. (Fabaceae), an ornamental flame tree, is traditionally used for infections and inflammatory conditions and its flowers are a rich source of flavonoids, tannins, and triterpenoids (Bhat et al., 2013; Bouayed & Piri, 2016). While each species individually has demonstrated antimicrobial activity in the literature, the combined, standardized action of these three plants as a single polyherbal preparation has not previously been systematically characterized.

The panel of test organisms selected for antimicrobial evaluation reflects pathogens of substantial clinical importance. Staphylococcus aureus and Enterococcus faecalis represent Gram-positive organisms with well-documented intrinsic and acquired resistance mechanisms, including methicillin resistance and vancomycin resistance, respectively (Tong et al., 2015; Arias & Murray, 2012). Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa represent Gram-negative organisms responsible for a large share of nosocomial and community-acquired infections; K. pneumoniae is notable for its propensity to acquire ESBL and carbapenemase resistance determinants (Paczosa & Mecsas, 2016), while P. aeruginosa

possesses intrinsic multidrug resistance mediated by low outer-membrane permeability, constitutive and inducible efflux pump systems, and inducible chromosomal AmpC β-lactamase (Lister et al., 2009). Candida albicans and Aspergillus niger were included as clinically important fungal pathogens amid rising rates of azole resistance (Pappas et al., 2016; Jeffery-Smith et al., 2018; Latgé & Chamilos, 2019). Bacillus subtilis was included as a standard Gram-positive reference organism for antimicrobial screening (Earl et al., 2008).

Despite the promise of polyherbal antimicrobials, relatively few published studies combine a rigorous, guideline-compliant safety evaluation with a comprehensive, multi-organism antimicrobial characterization of a standardized formulation. The present study was therefore designed to (i) prepare and phytochemically standardize a PHF of P. urinaria, M. koenigii, and D. regia by successive solvent extraction, qualitative phytochemical screening, and TLC fingerprinting; (ii) establish its acute oral safety profile in accordance with OECD Guideline 420; and (iii) characterize its antimicrobial spectrum, potency, and mode of action (static versus cidal) against a panel of six bacterial and two fungal strains of clinical relevance, benchmarked against ciprofloxacin and ketoconazole.

2. Materials and Methods

2.1 Plant Material Collection, Authentication, and Crude Drug Preparation

The whole aerial parts of Phyllanthus urinaria L., the leaves of Murraya koenigii (L.) Spreng., and the flowers of Delonix regia (Boj. ex Hook.) Raf. were collected during the appropriate harvesting season to ensure maximal phytochemical content. Taxonomic identification and authentication of each species were performed by a qualified taxonomist at the Botanical Survey of India (BSI), and voucher specimens were deposited in the institutional herbarium. Collected material was washed, shade-dried at 25 ± 2 °C to constant weight (10–21 days, depending on species), and reduced to a coarse powder that was passed through a 40-mesh BSS sieve (particle size ≈ 425 µm). Moisture content, total ash, acid-insoluble ash, and water-soluble ash of each powdered material were determined in triplicate as per Indian Pharmacopoeia/WHO methods for herbal quality control (WHO, 2011).

2.2 Preparation of the Polyherbal Formulation and Successive Solvent Extraction

The polyherbal formulation (PHF) was prepared by mixing equal proportions (w/w) of the individually dried and powdered plant materials. The mixed powder (250 g per batch, in triplicate) was subjected to successive solvent extraction using five solvents of increasing polarity — petroleum ether, ethyl acetate, acetone, ethanol, and hydroalcoholic solvent (ethanol:water, 70:30 v/v). Ethanol extraction was carried out by Soxhlet apparatus (48–72 h continuous hot reflux, ethanol ≥ 99.5%, Merck India); the remaining four solvents were applied sequentially to the same marc by cold maceration (7 days each, room temperature, with twice-daily agitation). Each filtrate was concentrated under reduced pressure (Büchi R-300 rotary evaporator, ≤ 40–45 °C) and dried to constant weight in a vacuum desiccator. Percentage yield was calculated as (weight of dried extract / weight of initial dried powder) × 100. Extracts were stored in amber glass vials at 2–8 °C until use.

2.3 Preliminary Phytochemical Screening

Each of the five solvent extracts was reconstituted (≈ 10 mg/mL) and screened qualitatively, in triplicate, for the major classes of secondary metabolites using standard colorimetric, turbidimetric, and precipitation reactions: alkaloids (Mayer's, Dragendorff's, Wagner's, Hager's tests), glycosides (Keller-Killiani, Borntrager's tests), flavonoids (alkaline reagent, lead acetate, Shinoda tests), triterpenoids and steroids (Liebermann-Burchard, Salkowski tests), tannins (ferric chloride, lead acetate, gelatin, potassium dichromate tests), saponins (froth and haemolysis tests), phenolics (ferric chloride, iodine tests), proteins/amino acids (biuret, Millon's, xanthoproteic, ninhydrin tests), and carbohydrates (Molisch's, Fehling's, Benedict's, Barfoed's tests). Results were graded semi-quantitatively as absent (−), present (+), moderately present (++), or strongly present (+++).

2.4 Thin-Layer Chromatographic (TLC) Fingerprinting

Each extract was chromatographed on pre-coated silica gel 60 F_{254} aluminium plates using a solvent system optimized for its polarity (toluene:ethyl acetate 9:1 for petroleum ether; chloroform:methanol 9:1 for ethyl acetate; chloroform:methanol 8:2 for acetone; ethyl acetate:formic acid:acetic acid:water 100:11:11:26 for ethanol; and butanol:acetic acid:water 4:1:5, upper layer, for the hydroalcoholic extract), developed in a pre-saturated chamber at 25 ± 2 °C. Resolved spots were visualized under UV light (254 and 365 nm) and after derivatization with anisaldehyde-sulphuric acid, vanillin-sulphuric acid, NP/PEG (flavonoid-specific), Dragendorff's (alkaloid-specific), and ferric chloride (phenolic/tannin-specific) spray reagents. Retardation factors (Rf) were calculated as the ratio of the distance travelled by the spot to that travelled by the solvent front.

2.5 Acute Oral Toxicity Study

Acute oral toxicity of the PHF was evaluated in adult female Wistar albino rats (8–12 weeks, 180–220 g) according to OECD Guideline No. 420 (Fixed Dose Procedure), following approval by the Institutional Animal Ethics Committee and in compliance with CPCSEA guidelines. The extract, suspended in 0.5% w/v sodium carboxymethylcellulose, was administered as a

single oral dose of 2000 mg/kg body weight (10 mL/kg dose volume) by gavage to three animals, with one additional vehicle-treated control animal. Animals were observed continuously for the first 30 minutes, periodically at 1, 2, 4, 8, and 24 h, and then once daily for 14 days for mortality, behavioural and autonomic signs, and body weight (Days 0, 7, 14) and food/water intake. At study termination, all animals underwent gross necropsy with macroscopic examination of major organs.

2.6 Test Microorganisms

Antimicrobial activity was evaluated against a panel of eight American Type Culture-equivalent reference strains obtained from the Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India, comprising three Gram-positive bacteria, three Gram-negative bacteria, and two fungal strains.

2.7 Antimicrobial Susceptibility Testing — Agar Well Diffusion

Antimicrobial screening was performed by the agar well diffusion method on Mueller-Hinton agar (bacteria) or Sabouraud dextrose agar (fungi) plates uniformly inoculated with standardized inoculum (0.5 McFarland; ≈ 1–2 × 10^6 CFU/mL for bacteria, ≈ 1–5 × 10^6 CFU or spores/mL for fungi). Wells (6 mm diameter) were punched and filled with 50 µL of PHF solution at 50, 100, 200, or 500 µg/mL (prepared in DMSO, final well concentration of DMSO ≤ 1% v/v). Plates were pre-diffused at 4 °C for 2 h and then incubated under organism-specific conditions (37 ± 1 °C, 18–24 h for bacteria; 35 ± 2 °C, 24–48 h for C. albicans; 25 ± 2 °C, 48–72 h for A. niger). Zones of inhibition were measured to the nearest 0.1 mm using a HiAntibiotic zone scale. Ciprofloxacin (5 µg disc) and ketoconazole (50 µg disc) served as positive controls for bacteria and fungi, respectively, and 1% DMSO served as the negative control. All determinations were performed in triplicate on three independent occasions.

2.8 Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by the broth micro-dilution method in 96-well microtitre plates following CLSI guidelines (M07-A10 for bacteria; M27-A3 for yeasts; M38-A2 for moulds). A two-fold serial dilution of the PHF (final well concentrations 7.81–1000 µg/mL) was prepared in Mueller-Hinton Broth or Sabouraud Dextrose Broth and inoculated with the standardized microbial suspension. Following incubation under organism-specific conditions, growth was assessed visually for turbidity and confirmed with a 0.01% resazurin colorimetric indicator (blue-to-pink colour change denoting viable growth). MIC was defined as the lowest concentration producing no visible turbidity or colour change; the modal value across three independent triplicate determinations was reported.

2.9 Determination of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

From each clear MIC well and the well immediately above the MIC, 10 µL aliquots were sub-cultured onto drug-free Mueller-Hinton agar or Sabouraud dextrose agar by the four-quadrant streak method and incubated under organism-specific conditions. MBC/MFC was defined as the lowest concentration yielding no colony growth (≥ 99.9% kill). The MBC/MIC (or MFC/MIC) ratio was used to classify the formulation as bactericidal/fungicidal (ratio ≤ 4) or bacteriostatic/fungistatic (ratio > 4).

2.10 Statistical Analysis

Data are expressed as mean ± standard deviation (n = 3). Normality and homogeneity of variance were assessed by the Shapiro-Wilk and Levene's tests, respectively. Group comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post-hoc test, with the Kruskal-Wallis test and Dunn's post-hoc test applied where the assumptions of parametric testing were not met. Concentration–response relationships (zone of inhibition versus log concentration) were assessed by linear regression. A p-value < 0.05 was considered statistically significant. Analyses were performed using GraphPad Prism v10.0 and IBM SPSS Statistics v26.0.

3. Results

3.1 Physicochemical Characterization of Plant Materials

Moisture content of the three powdered plant materials ranged from 7.85 ± 0.28% (M. koenigii) to 9.14 ± 0.35% (D. regia), well within WHO limits for crude drug materials. Total ash values (10.83–12.56%) and acid-insoluble ash values (2.67–3.24%) confirmed adequate purity and minimal extraneous mineral contamination across all three species (Table 1).

Table 1. Physicochemical Parameters of the Powdered Plant Materials (Mean ± SD, n = 3)

Parameter	Phyllanthus urinaria	Murraya koenigii	Delonix regia
Moisture content (% w/w)	8.42 ± 0.31	7.85 ± 0.28	9.14 ± 0.35
Total ash value (% w/w)	12.56 ± 0.42	10.83 ± 0.37	11.72 ± 0.44
Acid-insoluble	3.24 ± 0.18	2.67 ± 0.15	3.08 ± 0.21

Parameter	Phyllanthus urinaria	Murraya koenigii	Delonix regia
ash (% w/w)			
Water-soluble ash (% w/w)	6.73 ± 0.25	5.42 ± 0.22	6.18 ± 0.29
Foreign organic matter (% w/w)	0.42 ± 0.08	0.35 ± 0.06	0.58 ± 0.12
Swelling index	3.2 ± 0.4	2.8 ± 0.3	3.6 ± 0.5
Haemolytic activity index	1.8 ± 0.2	1.5 ± 0.2	2.1 ± 0.3

IP = Indian Pharmacopoeia; WHO = World Health Organization.

3.2 Percentage Yield of Successive Solvent Extracts

Cumulative extraction yield across all five solvents was 36.77 ± 0.59% w/w. Yield increased progressively with solvent polarity, from 2.33 ± 0.10% (petroleum ether) to 14.10 ± 0.31% (hydroalcoholic), reflecting the predominantly polar nature of the formulation's phytoconstituents (Table 2).

Table 2. Percentage Yield and Physical Characteristics of Successive Solvent Extracts

Solvent	Polarity Index	Extraction Method	Yield (% w/w)	Physical Appearance
Petroleum ether	0.1	Cold maceration	2.33 ± 0.10	Dark greenish-brown, oily semi-solid
Ethyl acetate	4.4	Cold maceration	3.79 ± 0.15	Yellowish-brown, amorphous powder
Acetone	5.1	Cold maceration	5.05 ± 0.18	Brown, sticky semi-solid
Ethanol	5.2	Soxhlet extraction	11.50 ± 0.25	Dark brown, sticky resinous mass
Hydroalcoholic (70:30)	≈ 8.0	Cold maceration	14.10 ± 0.31	Dark reddish-brown, viscous liquid
Total	—	—	36.77 ± 0.59	—

Values expressed as mean ± SD (n = 3); 250 g powdered plant material extracted per batch.

3.3 Preliminary Phytochemical Screening

Flavonoids, tannins, and phenolics were the most abundant and widely distributed phytoconstituent classes, present strongly (+++) in both the ethanol and hydroalcoholic extracts. Alkaloids were detected in the acetone, ethanol, and hydroalcoholic fractions, with the strongest response in ethanol. Triterpenoids and steroids were confined mainly to the non-polar fractions, consistent with their lipophilic character. The ethanol and hydroalcoholic extracts possessed the broadest

phytochemical spectrum, with 9 and 10 of the 10 tested classes detected, respectively (Table 3).

Table 3. Qualitative Phytochemical Screening of the Polyherbal Formulation Extracts

Phytoconstituent Class	Pet. Ether	Ethyl Acetate	Acetone	Ethanol	Hydroalcoholic
Alkaloids	−	−	+	++	+
Glycosides	−	+	+	++	++
Flavonoids	−	++	++	+++	+++
Triterpenoids	++	+	+	+	−
Steroids / Phytosterols	++	+	+	−	−
Tannins	−	+	++	+++	+++
Saponins	−	−	+	++	++
Phenolics	−	+	++	+++	+++
Proteins	−	−	−	+	++
Carbohydrates	−	−	−	+	++

(+++) Strongly present; (++) moderately present; (+) present; (−) absent.

3.4 TLC Fingerprinting

The number of resolved TLC spots increased progressively with solvent polarity, from 4 spots in the petroleum ether extract to a maximum of 9 spots in the ethanol extract, before declining slightly to 8 spots in the hydroalcoholic extract. NP/PEG-reactive (flavonoid) spots were detected in all extracts except petroleum ether; Dragendorff's-reactive (alkaloid) spots were confined to the ethanol and hydroalcoholic extracts, corroborating the phytochemical screening results (Table 4).

Table 4. Summary of TLC Fingerprint Data of the Polyherbal Formulation Extracts

Extract	Solvent System (v/v)	No. of Spots	Rf Range	Principal Detection Response
Petroleum ether	Toluene:Ethyl acetate (9:1)	4	0.12–0.82	UV-quenching; grey-blue with anisaldehyde-H_2SO_4 (terpenoids/sterols)
Ethyl acetate	Chloroform: Methanol (9:1)	6	0.08–0.78	Yellow-green fluorescence, NP/PEG (flavonoids); violet, vanillin-H_2SO_4
Acetone	Chloroform: Methanol (8:2)	7	0.06–0.81	Yellow-orange fluorescence, NP/PEG; blue-black, $FeCl_3$ (phenolics/tannins)
Ethanol	EtOAc:Formic acid:Acetic acid:Water (100:11:11:26)	9	0.05–0.88	Orange-yellow, NP/PEG; orange, Dragendorff's

Extract	Solvent System (v/v)	No. of Spots	Rf Range	Principal Detection Response
				(alkaloids); green-black, $FeCl_3$
Hydroalcoholic	Butanol:Acetic acid:Water (4:1:5)	8	0.09–0.85	Yellow, NP/PEG; orange, Dragendorff's; blue-violet, $FeCl_3$

NP/PEG = natural product–polyethylene glycol reagent (flavonoid-specific); $FeCl_3$ = ferric chloride reagent (phenolic/tannin-specific).

3.5 Acute Oral Toxicity

No mortality, behavioural or autonomic abnormalities, or signs of overt toxicity were observed in any animal throughout the 14-day observation period following a single oral dose of 2000 mg/kg. All animals showed steady, physiologically normal body-weight gain, from a mean of 192.47 ± 5.80 g on Day 0 to 205.97 ± 5.83 g on Day 14 (+7.02%), with food and water intake remaining within normal historical ranges throughout the study. Gross necropsy revealed no macroscopic abnormalities in the liver, kidneys, spleen, heart, lungs, gastrointestinal tract, or other examined organs in either treated or vehicle-control animals. On this basis, the oral LD_{50} of the polyherbal formulation was estimated to exceed 2000 mg/kg body weight, placing it in GHS Category 5 (or "not classified"), the lowest acute oral toxicity hazard category.

3.6 Antimicrobial Susceptibility: Zone of Inhibition (Agar Well Diffusion)

The polyherbal formulation produced clear, concentration-dependent zones of inhibition against all eight test organisms, with zone diameters increasing progressively across the tested range of 50–500 µg/mL (linear regression of zone diameter versus log concentration, R^2 = 0.96–0.99 for all organisms). Among Gram-positive bacteria, Bacillus subtilis was the most susceptible (21.4 ± 0.5 mm at 500 µg/mL), followed by Staphylococcus aureus (19.8 ± 0.6 mm) and Enterococcus faecalis (17.6 ± 0.5 mm). Among Gram-negative bacteria, Escherichia coli was the most susceptible (16.6 ± 0.5 mm), followed by Klebsiella pneumoniae (15.2 ± 0.6 mm) and Pseudomonas aeruginosa (14.2 ± 0.6 mm), the latter consistent with its well-characterized intrinsic multidrug resistance phenotype. Among fungi, Candida albicans (18.6 ± 0.5 mm) was more susceptible than Aspergillus niger (15.8 ± 0.6 mm). The 1% DMSO negative control produced no zone of inhibition against any organism (Table 5, Table 6).

Table 5. Test Microorganisms and Zone of Inhibition Produced by the Polyherbal Formulation (Agar Well Diffusion)

Organism (Strain)	Type	50 µg/mL	100 µg/mL	200 µg/mL	500 µg/mL	Standard[a] (mm)	1% DMSO
Bacillus subtilis (MTCC 441)	G+ bacterium	9.2± 0.4	13.6± 0.5	17.8± 0.6	21.4± 0.5	28.6 ±0.8	0.0
Staphylococcus aureus (MTCC 96)	G+ bacterium	8.4± 0.5	12.2± 0.4	16.4± 0.5	19.8± 0.6	26.4 ±0.7	0.0
Enterococcus faecalis (MTCC 439)	G+ bacterium	7.6± 0.5	10.8± 0.6	14.2± 0.5	17.6± 0.5	22.8 ±0.6	0.0
Pseudomonas aeruginosa (MTCC 424)	G− bacterium	6.2± 0.4	8.4 ±0.5	11.6± 0.5	14.2± 0.6	24.2 ±0.7	0.0
Escherichia coli (MTCC 443)	G− bacterium	7.4± 0.5	10.6± 0.5	13.8± 0.6	16.6± 0.5	25.8 ±0.6	0.0

Organism (Strain)	Type	50 µg/mL	100 µg/mL	200 µg/mL	500 µg/mL	Standard[a] (mm)	1% DMSO
Klebsiella pneumoniae (MTCC 109)	G– bacterium	6.8± 0.4	9.2 ±0.6	12.4± 0.5	15.2± 0.6	23.4 ±0.8	0.0
Candida albicans (MTCC 227)	Fungus (yeast)	7.8± 0.4	11.4± 0.5	15.2± 0.6	18.6± 0.5	24.6 ±0.7	0.0
Aspergillus niger (MTCC 281)	Fungus (mould)	6.4± 0.5	9.6 ±0.5	12.8± 0.6	15.8± 0.6	22.2 ±0.6	0.0

Values are mean ± SD zone of inhibition in mm (n = 3; well diameter 6 mm). [a]Standard = ciprofloxacin (5 µg disc) for bacteria, ketoconazole (50 µg disc) for fungi. MTCC = Microbial Type Culture Collection.

3.7 Minimum Inhibitory, Bactericidal, and Fungicidal Concentrations

MIC values of the polyherbal formulation ranged from 62.5 µg/mL (B. subtilis) to 250 µg/mL (P. aeruginosa, E. coli, K. pneumoniae, and A. niger), consistent with a moderate-to-good level of antimicrobial potency for a crude, multi-component plant extract. MBC/MFC values ranged from 125 to 1000 µg/mL, and the resulting MBC/MIC (or MFC/MIC) ratios were ≤ 4 for every organism tested — the threshold below which an agent is classified as cidal rather than static — indicating that the polyherbal formulation is bactericidal against all six bacterial strains and fungicidal against both fungal strains (Table 6).

Table 6. MIC, MBC/MFC, and Classification of Antimicrobial Activity of the Polyherbal Formulation

Organism	MIC (µg/mL)	MIC, Ciprofloxacin	MIC, Ketoconazole	MBC/MFC (µg/mL)	MBC (MFC)/MIC Ratio	Classification
Bacillus subtilis	62.5	0.06	—	125	2.0	Bactericidal
Staphylococcus aureus	125	0.12	—	250	2.0	Bactericidal
Enterococcus faecalis	125	0.25	—	500	4.0	Bactericidal
Pseudomonas aeruginosa	250	0.12	—	1000	4.0	Bactericidal
Escherichia coli	250	0.06	—	500	2.0	Bactericidal
Klebsiella pneumoniae	250	0.25	—	1000	4.0	Bactericidal
Candida albicans	125	—	0.25	250	2.0	Fungicidal
Aspergillus niger	250	—	0.50	500	2.0	Fungicidal

MIC/MBC/MFC values are modal values from broth micro-dilution and sub-culture (n = 3 independent triplicates). MBC(MFC)/MIC ≤ 4 = bactericidal/fungicidal; > 4 = bacteriostatic/fungistatic.

3.8 Comparative Activity Relative to Reference Standards

At 500 µg/mL, the polyherbal formulation exhibited 58.7–77.2% of the zone-of-inhibition activity produced by the reference standards across the eight test organisms, with a mean relative

activity of 70.2%. The highest relative activity was observed against Enterococcus faecalis (77.2% of ciprofloxacin) and Candida albicans (75.6% of ketoconazole), and the lowest against Pseudomonas aeruginosa (58.7% of ciprofloxacin) (Table 7).

Table 7. Antimicrobial Activity of the Polyherbal Formulation (500 µg/mL) Relative to Reference Standards

Organism	ZOI, PHF 500 µg/mL (mm)	ZOI, Reference Standard (mm)	Relative Activity (%)
Bacillus subtilis	21.4 ± 0.5	28.6 ± 0.8 (Ciprofloxacin)	74.8
Staphylococcus aureus	19.8 ± 0.6	26.4 ± 0.7 (Ciprofloxacin)	75.0
Enterococcus faecalis	17.6 ± 0.5	22.8 ± 0.6 (Ciprofloxacin)	77.2
Pseudomonas aeruginosa	14.2 ± 0.6	24.2 ± 0.7 (Ciprofloxacin)	58.7
Escherichia coli	16.6 ± 0.5	25.8 ± 0.6 (Ciprofloxacin)	64.3
Klebsiella pneumoniae	15.2 ± 0.6	23.4 ± 0.8 (Ciprofloxacin)	65.0
Candida albicans	18.6 ± 0.5	24.6 ± 0.7 (Ketoconazole)	75.6
Aspergillus niger	15.8 ± 0.6	22.2 ± 0.6 (Ketoconazole)	71.2

ZOI = zone of inhibition; PHF = polyherbal formulation. Mean relative activity across all eight organisms = 70.2%.

4. Discussion

This study provides the first systematic phytochemical and antimicrobial characterization of a polyherbal formulation combining P. urinaria, M. koenigii, and D. regia. Three findings merit particular emphasis: the concentration-dependent, broad-spectrum antimicrobial activity of the formulation; its consistently bactericidal/fungicidal mode of action; and its favourable acute safety profile, which together support the biological plausibility of the traditional antimicrobial use of these plants.

The phytochemical screening and TLC fingerprinting results provide a mechanistic basis for the observed activity. Flavonoids, tannins, and phenolics were the most abundant and widely distributed constituent classes, and each has well-documented antimicrobial mechanisms: flavonoids disrupt microbial membranes and inhibit key enzymes such as DNA gyrase and topoisomerase IV; tannins complex with microbial surface proteins and enzymes and chelate metal ions essential for microbial metabolism; and phenolics act as antioxidants, enzyme inhibitors, and membrane-disrupting agents (Cowan, 1999; Gyawali & Ibrahim, 2014). The carbazole alkaloids characteristic of M. koenigii and the hydrolysable tannins characteristic of P. urinaria likely contribute disproportionately to the antimicrobial effect, consistent with prior reports on the individual species (Nagappan et al., 2017; Liu et al., 2014). The progressive increase in the number of resolved TLC spots and in extraction yield from non-polar to polar solvents — culminating in the ethanol and hydroalcoholic fractions — corroborates the phytochemical screening and indicates that these two extracts carry the greatest phytochemical diversity and, by extension, the greatest antimicrobial potential of the five fractions examined.

The differential susceptibility observed between Gram-positive and Gram-negative bacteria is consistent with well-established differences in cell-envelope architecture. The outer membrane of Gram-negative bacteria, containing lipopolysaccharide, acts as a permeability barrier that restricts entry of large, hydrophobic, or charged phytoconstituents, while porins permit passage only of relatively small, hydrophilic molecules (typically < 600 Da) (Lister et al., 2009). This structural barrier plausibly explains the smaller zones of inhibition and higher MIC values observed for the Gram-negative organisms relative to the Gram-positive organisms. Despite this, the formulation retained meaningful activity against Pseudomonas aeruginosa (MIC 250 µg/mL, MBC/MIC ratio 4.0), an organism with pronounced intrinsic multidrug resistance mediated by efflux pumps, low outer-membrane permeability, and inducible AmpC β-lactamase — a result that is clinically noteworthy given the limited therapeutic options for P. aeruginosa infections.

The consistently cidal mode of action (MBC/MFC-to-MIC ratio ≤ 4 for all eight organisms) is a therapeutically significant finding, as bactericidal and fungicidal agents are generally preferred over static agents for serious infections, particularly in immunocompromised patients, because they eliminate rather than merely suppress the pathogen, reducing the risk of relapse on treatment discontinuation. The antifungal activity against Candida albicans and, to a lesser extent, Aspergillus niger is noteworthy in light of the increasing global incidence of azole-resistant

Candida species and the limited antifungal armamentarium (Jeffery-Smith et al., 2018).

While the MIC values of the polyherbal formulation (62.5–250 µg/mL) were, as expected, several hundred- to several thousand-fold higher than those of the purified reference antibiotics, this comparison should be interpreted with appropriate context. Crude plant extracts are complex mixtures in which the active antimicrobial constituents represent only a fraction of the total material; the effective concentration of these active principles is accordingly diluted relative to a purified single-molecule drug. MIC values in the 62.5–500 µg/mL range are generally regarded in the phytochemical literature as indicative of moderate-to-good antimicrobial activity for a crude extract, and the mean relative activity of 70.2% compared with established antibiotics at equivalent test concentrations further underscores the pharmacological significance of the formulation. Bioassay-guided fractionation would be expected to substantially lower the MIC of the isolated active constituents.

The favourable acute safety profile observed here (no mortality or toxic signs at 2000 mg/kg; estimated LD_{50} > 2000 mg/kg) is consistent with the individually reported safety of the constituent plants: M. koenigii is widely consumed as a culinary herb with a documented favourable toxicity profile at doses up to 2000 mg/kg, P. urinaria has a long history of traditional use without reports of significant adverse effects, and D. regia flower extracts have been reported to have an LD_{50} exceeding 2000 mg/kg in Wistar rats. This safety margin, combined with the demonstrated antimicrobial efficacy, supports the biological rationale for further pharmaceutical development of the formulation. Acute toxicity data alone, however, do not capture chronic, organ-specific, or genotoxic risk, and studies of this kind should be regarded as a necessary but not sufficient step toward clinical translation.

This study has several limitations. First, the antimicrobial activity was characterized for the crude polyherbal extract rather than isolated active constituents, so the specific compounds responsible for the observed effects, and their individual potencies, remain to be established through bioassay-guided fractionation. Second, the mechanism of action was inferred from the known pharmacology of the dominant phytoconstituent classes rather than demonstrated directly through, for example, membrane integrity assays, enzyme inhibition assays, or electron microscopy. Third, activity was assessed against a defined panel of eight reference strains; extension to clinical multidrug-resistant isolates, biofilm-forming strains, and a broader range of pathogens would strengthen the clinical relevance of these findings. Fourth, in vivo efficacy in an infection model has not yet been established. Addressing these limitations represents a logical next phase of this research programme.

5. Conclusion

A polyherbal formulation combining Phyllanthus urinaria, Murraya koenigii, and Delonix regia, standardized by successive solvent extraction, phytochemical screening, and TLC fingerprinting, exhibited concentration-dependent, broad-spectrum, and predominantly bactericidal/fungicidal activity against a panel of clinically relevant Gram-positive bacteria, Gram-negative bacteria, and fungi, including organisms with documented intrinsic multidrug resistance. This activity was achieved within a formulation shown to possess a wide margin of acute oral safety (LD_{50} > 2000 mg/kg). Collectively, these findings provide a scientific rationale for the traditional antimicrobial use of these three plants and support the further development of this polyherbal formulation as a standardized, evidence-based botanical antimicrobial candidate, pending bioassay-guided fractionation, mechanistic elucidation, and in vivo efficacy studies.

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