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Research Article
Phytochemical Analysis and Evaluation of the Antimicrobial and Cytotoxic Activities of *Rothia indica* (L.) Druce Leaf Extracts

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Abstract

Background and Objective: The exploration of bioactive compounds in medicinal plants has received considerable attention in recent years. *Rothia indica* is one such plant that requires further investigation to determine its phytochemical constituents and therapeutic properties. This study investigated the phytochemical constituents, antimicrobial activity and cytotoxic potential of different solvent extracts of *Rothia indica* leaves. **Materials and Methods:** Leaf powder was extracted using petroleum ether, ethyl acetate, ethanol and aqueous solvents using the Soxhlet extraction method and the major phytochemicals were tested. Antimicrobial activity was evaluated at the concentration of 25 µg, 50 µg and 100 µg against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae* using the agar-well diffusion method. Cytotoxicity was determined using the brine shrimp lethality assay at concentration of 1, 10, 100 and 1000 ppm to determine the LC_{50} value. All the experiments were performed in triplicate (n = 3). Data were expressed as Mean±SD and analysed using One-way ANOVA followed by Tukey's HSD *post hoc* test in IBM SPSS Statistics, with $p<0.05$ considered statistically significant. **Results:** Phytochemical screening revealed the presence of alkaloids, flavonoids, phenolics, tannins, steroids and terpenoids, with the ethanol extract showing greater bioactive content. The ethanol extract exhibited notable antibacterial activity, with inhibition zones of 8-10 mm and a maximum cytotoxic effect, with an LC_{50} value of 1.38 ppm. The observed differences among the extracts were statistically significant ($p<0.05$). **Conclusion:** *Rothia indica* leaves, particularly their ethanol extract, exhibit significant antimicrobial and cytotoxic properties, indicating their potential as a promising source of therapeutic bioactive compounds for pharmaceutical applications.

Key words: *Rothia indica* leaves, bioactive compounds, antimicrobial activity, brine shrimp lethality assay, cytotoxicity

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Medicinal plants have long been recognised as valuable sources of bioactive compounds with significant therapeutic potential. Growing interest in plant-derived natural products is driven by their capacity to provide novel antimicrobial and anticancer agents, offering a promising strategy for combating drug-resistant pathogens and improving human health[1,2]. Despite these therapeutic benefits, some medicinal plants may also contain toxic constituents that can pose health risks if used improperly or without appropriate evaluation[3,4]. Therefore, evaluating both their pharmacological properties and safety is essential before their therapeutic application[5].

Phytochemicals are naturally occurring bioactive molecules found in all plant parts, such as roots, stems, leaves, flowers, fruits, seeds, bark and rhizomes[6,7]. Phytochemicals present in plants have several health advantages and are natural alternatives to synthetic pharmaceuticals.

The rapid growth and spread of multidrug-resistant (MDR) pathogenic microorganisms have rendered many existing antimicrobial medications ineffective[8,9]. Antimicrobial resistance (AMR) is recognized as one of the most serious global public health challenges[10]. This resistance has made the management and treatment of infectious diseases more difficult[11,12].

Furthermore, medicinal plant extracts have emerged as promising, affordable alternatives to conventional antimicrobial agents[13]. However, the antibacterial and antioxidant capabilities of many plant species have received little attention[12]. In recent years, there has been a notable increase in the screening of herbal extracts for pharmacological potential[14], which highlights the need for systematic phytochemical and antimicrobial research.

Cytotoxicity was assessed using the Brine Shrimp Lethality Test (BSLT), with *Artemia salina* serving as the test organism. It is normal practice to conduct preliminary tests on brine shrimp larvae to determine the anti-tumor and anti-cancer effectiveness of a substance[15]. Over the past 30 years, the Brine Shrimp Assay has been widely used to test the toxicity of a variety of plant products[5]. A BSLA can be used to assess the toxicity of various active compounds in plants. This method is very simple, inexpensive and attractive[16].

Among the several plant families, Fabaceae, also known as the legume, pea, bean, or pulse family, is one of the largest and most economically significant flowering plant families[17]. It is widely distributed and dominant in tropical rainforests and dry woods throughout Africa and America[18]. The genus *Rothia* (Fabaceae) contains only two known species, *Rothia indica* and *Rothia hirsute*[7,19]. *Rothia indica* is a prostrate annual herb that commonly grows in deciduous forests and plains. In India, it is distributed in Andhra Pradesh, Tamil Nadu, Karnataka, Kerala and Odisha.

Despite the traditional importance of *Rothia indica*, scientific information on its phytochemical composition, antimicrobial activity and cytotoxic potential remains limited. Therefore, the present study aimed to investigate the phytochemical constituents of petroleum ether, ethyl acetate, ethanol and aqueous leaf extracts of *Rothia indica* and to evaluate their antimicrobial and cytotoxic activities. It was anticipated that the solvent extracts of *Rothia indica* would differ in their phytochemical composition, antimicrobial activity and cytotoxic potential.

MATERIALS AND METHODS

Study area and duration: The study was conducted from November 2025 to February 2026. Fresh leaves of *Rothia indica* were collected from Coimbatore, Tamil Nadu, India, in November 2025. Phytochemical screening and brine shrimp cytotoxicity study were carried out in the Entomology research laboratory, Department of Zoology, Government Arts College, Coimbatore, Tamil Nadu, India. Antimicrobial assay was carried out in the Department of Biotechnology, Dr. NGP Arts and Science College, Coimbatore, Tamil Nadu, India.

Plant material collection and extract preparation: The collected plant material was authenticated at the Botanical Survey of India (BSI), Southern Region Centre, Coimbatore, by Dr. M. U. Sharief, Scientist 'F' & Head of office, Authentication No. BSI/SRC/5/23/2022/Tech-537. The collected *Rothia* leaves were thoroughly washed with tap water, shade-dried and ground into a fine powder. The powdered material was subjected to Soxhlet extraction using solvents of increasing polarity[20]. For 45 g of leaf powder was extracted successively with 450 mL of corresponding solvents (solvent to sample ratio 1:10 w/v). The extraction temperature was maintained close to the boiling point of each solvent and yields of extracts of petroleum ether (40°C-1.6 g), ethyl acetate (77°C-2.7 g), ethanol (78°C-5.2 g) and aqueous (100°C-7.1g) was obtained for 8 hrs. The obtained extract was concentrated using a rotary evaporator at approximately 38°C and stored at 4°C until further analysis[7].

Preliminary phytochemical screening: Qualitative phytochemical analysis of the petroleum ether, ethyl acetate, ethanol and aqueous extracts was performed using standard

procedures ures[21] to detect the presence of secondary metabolites such as Alkaloids, flavonoids, phenols, tannins, steroids, triterpenoids, cardiac glycosides, gums and mucilage, anthraquinones and Saponins.

Antimicrobial activity

Agar well diffusion method: The antibacterial activity of *Rothia indica* leaf extracts was evaluated using the agar well diffusion method[22]. The test organisms, including clinical isolates of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae*, were obtained from Kovai Medical Center and Hospital (KMCH), Coimbatore, Tamilnadu, India. The cultured was maintained on nutrient agar slants at 4°C in the Department of Biotechnology, Dr. NGP Arts and Science College, Coimbatore and the bacterial inoculum was standardized to 0.5 McFarl and standard before the antimicrobial assay.

Sterile Mueller-Hinton agar plates were prepared and uniformly inoculated with the standardized bacterial suspension using a sterile cotton swab. Wells of approximately 6 mm diameter were aseptically punched into the agar using a sterile cork borer. Different concentrations of plant extracts (25, 50 and 100 µg/mL) were added to the wells. Streptomycin was used as the positive control and the respective solvents served as the negative controls. The plates were incubated at 37°C for 24 hrs, after which the diameter of the zone of inhibition was measured in millimetres[22].

Statistical analysis: The zone of inhibition data were expressed as Mean±Standard Deviation (SD). Statistical analysis was performed using one-way analysis of variance, followed by Tukey's HSD *post hoc* test in IBM SPSS Statistics. Differences among treatments within each bacterial group were considered statistically significant at $p<0.05$.

Cytotoxicity study

Brine shrimp lethality assay

Hatching of eggs and bioassays: Brine shrimp eggs were collected from Beena Aquarium, Coimbatore, Tamil Nadu, India. Artificial seawater is used to hatch brine shrimp eggs, which were prepared with distilled water and NaCl. The pH was checked and adjusted to 8-8.5[16]. The eggs were hatched under continuous aeration and illumination at 25-28°C for 48 hrs. Hatched larvae were collected and separated. The bioassay was carried out using 24 hrs old brine shrimp nauplii.

A stock solution (1%) was prepared by dissolving 0.1 g of the plant extract in 10 mL of the respective solvents. From this stock, different concentrations (1, 10, 100 and 1000 ppm) were prepared by serial dilutions using artificial seawater. For the assay, 2 mL of each concentration was added to test tubes and the final volume was adjusted to 5 mL using artificial seawater. Ten Artemia salina nauplii were introduced into each tube. Each experiment was performed in triplicate to ensure accuracy. Artificial seawater containing Tween 80 (or solvent control) was used as the negative control and potassium dichromate served as the positive control[23]. After 24 hrs of incubation, the number of dead nauplii was counted and the percentage mortality was calculated[16].

Statistical analysis: Data were analyzed using the IBM SPSS Statistics software. The lethal concentration (LC_{50}) and lower and upper confidence limits (LCL-UCL) at 95% confidence interval were determined by probit analysis. Percentage mortality data were subjected to One-way Analysis of Variance (ANOVA), followed by Tukey's HSD *post hoc* test. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Preliminary screening of secondary metabolites: Preliminary phytochemical screening of *Rothia indica* leaf extracts revealed the presence of several bioactive secondary metabolites, which varied with solvent polarity. Among the extracts, ethanol showed the highest number of phytoconstituents, followed by ethyl acetate and aqueous extracts, while petroleum ether showed minimal activity (Table 1).

Alkaloids were detected mainly in ethanol and ethyl acetate extracts, whereas flavonoids and phenolic compounds were strongly present in the ethanol extract. Tannins were observed in both the ethanol and aqueous extracts, indicating their polar nature. Steroids and terpenoids were moderately present in the ethanol and ethyl acetate extracts. Cardiac glycosides were also detected in selected extracts, suggesting their pharmacological significance. However, saponins, gums, mucilage, fixed oils and anthraquinones were absent in all the extracts. Similar findings were observed in *Rothia indica* root and fruit extracts[7], which showed that steroids and cardiac glycosides were absent in root and fruit extracts but were highly present in leaf extracts.

These phytochemicals exhibit a wide range of biological activities, including antimicrobial, antioxidant, anti-inflammatory, cytotoxic and cardioprotective effects. In particular, phenolics and flavonoids contribute to antioxidant activity, alkaloids to antimicrobial and cytotoxic properties and terpenoids and steroids to anti-inflammatory actions. The higher concentration of these compounds in ethanol extract suggests its suitability for extracting pharmacologically active constituents[24-26].

Table 1: Qualitative phytochemical screening of Rothia indica leaf extracts

		Organic solvents			
Chemical constituent	Tests	PE	EA	ET	AQ
Alkaloids	a) Dragendorff's test	-	++	+++	+
	b) Mayer's test	-	+	++	+
	c) Wagner's test	-	-	-	-
	d) Hager's test	-	-	++	+
Flavonoids	10% HCl & 5% NaOH test	-	-	+++	+
Phenolics	5% $FeCl_3$ test	+	+++	+++	++
Tannin	Lead sub acetate test	-	-	+++	+++
Steroids	Liebermann-Burchard's test	+	++	+++	+
Terpenoids	a)Liebermann-Burchard's test	-	-	++	+
	b) Salkowski's test	-	-	-	-
Saponins	Foam test	-	-	-	-
Cardiac-Glycosides	Keller-Kiliani test	+	++	++	-
Gum & Mucilages	Whistler & BeMiller test	-	-	-	-
Fixed oils	Spot test	-	-	-	-
Anthraquinones	Sanker and Nahar test	-	-	-	-

PE-Petroleum Ether, EA- Ethyl Acetate, ET- Ethanol, AQ- Aqueous, (+) Present and (-) Absent

Table 2: Antimicrobial activity of Rothia indica leaf extracts against selected bacterial pathogens measured as zone of inhibition (mm)

Organism	Extract	Streptomycin (mm)	25 µg (mm)	50 µg (mm)	100 µg (mm)
Escherichia coli (Gram-negative)	RI.L.PE	11± 0.5^a	ND	ND	ND
	RI.L.EA	11± 0.2^a	ND	9±0.9^a	8±0.1^a
	RI.L.ET	10± 0.0^b	ND	5±1^b	8±1^a
	RI.L.AQ	11±0.7^a	ND	ND	ND
Pseudomonas aeruginosa (Gram-negative)	RI.L.PE	10±0.2^a	ND	ND	ND
	RI.L.EA	10±0.5^a	2±0.6^b	5±1.2^b	5±0.4^b
	RI.L.ET	10±0.0^a	7±1^a	10±1^a	9±0.3^a
	RI.L.AQ	10±0.2^a	ND	3±0.7^c	5±0.1^b
Staphylococcus aureus (Gram-positive)	RI.L.PE	9±0.7^b	ND	ND	1±0.0^d
	RI.L.EA	9±0.7^b	ND	ND	8±0.1^c
	RI.L.ET	9±0.1^b	3±0.9^a	6±0.4^a	10±0.7^b
	RI.L.AQ	10±1.0^a	ND	6±0.3^a	11±0.3^a
Klebsiella pneumonia (Gram-negative)	RI.L.PE	11±0.1^a	ND	ND	ND
	RI.L.EA	9±0.1^b	ND	3±0.7^b	5±0.1^b
	RI.L.ET	11±0.3^a	2±0.4^a	6±0.9^a	8±0.2^a
	RI.L.AQ	11±0.2^a	ND	ND	3±0.1^c

Values are expressed as Mean±SD (n = 3) of the inhibition zone diameter (mm), Different superscript letters within each bacterial species indicate statistically significant differences at $p<0.05$ (one-way ANOVA followed by Tukey's HSD test using IBM SPSS Statistics), RI.L.PE-petroleum ether extract, RI.L.EA-ethyl acetate extract, RI.L.ET- ethanol extract, RI.L.AQ- aqueous extract (n = 3), 'ND' No detectable zone of inhibition and Streptomycin (100 µg) was used as positive control

Antimicrobial activity: The antimicrobial activity of petroleum ether, ethyl acetate, ethanol and aqueous leaf extracts of Rothia indica was evaluated against selected Gram-positive and Gram-negative bacterial strains using the agar well diffusion method. The test organisms included *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. *Streptomycin* was used as the positive control, while the corresponding extraction solvent served as the negative control. Antibacterial activity was assessed by measuring the diameter of the zones of inhibition (mm) following treatment with extract concentrations of 25, 50 and 100 µg per well. All experiments were performed in triplicate the results were expressed as Mean±Standard Deviation.

Among all the extracts, the ethanol extract (RIL.ET) exhibited the highest antimicrobial activity across all tested organisms, with zones of inhibition ranging from 5-10 mm in *E. coli*, 7-10 mm in *P. aeruginosa*, 3-10 mm in *S. aureus* and 2-8 mm in *K. pneumoniae* (Table 2). This indicates the broad-spectrum efficacy of the model. The ethyl acetate extract (RIL.EA) exhibited moderate activity, particularly against *E. coli* (up to 9 mm), *P. aeruginosa* (up to 5 mm), *S. aureus* (8 mm) and *K. pneumoniae* (5 mm), suggesting the presence of semi-polar antimicrobial compounds. The aqueous extract (RIL.AQ) demonstrated selective activity, with the highest inhibition observed against *S. aureus* (11 mm at 100 µg), followed by moderate activity against *P. aeruginosa* (3-5 mm) and minimal effect on *K. pneumoniae* (3 mm). No activity was

Table 3: Percentage mortality and LC_{50} values of different solvent extracts of *Rothia indica* leaf against *Artemia salina* nauplii

Treatments	Concentration (ppm)	Total nauplii	Number of dead Nauplii R1	R2	R3	Mortality±SD (%)	LC_{50} (ppm) (LCL-UCL)
Negative control (TWEEN 80)	-	30	0	0	0	0±0	0
Positive control	1	30	7	10	9	86.7±1.5^{c}	0.032 (0.00-2.94)
(potassium dichromate)	10	30	9	10	9	93.4±0.5^{b}	
	100	30	10	10	9	96.7±0.5^{a}	
	1000	30	10	10	9	96.7±0.5^{a}	
Petroleum ether	1	30	0	0	1	03.4±0.5^{d}	17.39 (3.31-91.20)
	10	30	1	1	0	06.7±0.5^{c}	
	100	30	2	2	1	16.7±0.5^{b}	
	1000	30	2	2	3	23.3±0.5^{a}	
Ethyl acetate	1	30	2	0	3	16.7±1.5^{d}	2.56 (1.71-3.83)
	10	30	2	4	5	36.7±1.5^{c}	
	100	30	5	3	6	46.6±1.5^{b}	
	1000	30	7	6	7	66.7±0.5^{a}	
Ethanol	1	30	2	3	1	20.0±1.0^{d}	1.38 (1.07-1.77)
	10	30	5	6	4	50.0±1.0^{c}	
	100	30	7	6	7	66.7±0.5^{b}	
	1000	30	10	9	9	93.4±0.5^{a}	
Aqueous	1	30	1	0	0	03.4±0.5^{d}	3.71 (2.75-5)
	10	30	3	1	1	16.7±1.1^{c}	
	100	30	4	2	0	20.0±2.0^{b}	
	1000	30	6	8	7	70.0±1.0^{a}	

Values are expressed as Mean±SD of triplicates. Different superscript letters within each solvent indicates statistically significant differences at $p<0.05$ (one-way ANOVA followed by Tukey HSD test using IBM SPSS statistics). LC_{50}-Lethal Concentration 50%, LCL- Lower Confidence Limit, UCL- Upper Confidence Limit, R1-Replication 1, R2-Replication 2 and R3-Replication 3

observed against *E. coli*. The petroleum ether extract (RIL.PE) showed negligible or no antimicrobial activity against most organisms, indicating that nonpolar compounds contribute minimally to antibacterial effects. Overall, Gram-positive bacteria (*S. aureus*) showed greater susceptibility, whereas Gram-negative bacteria (*E. coli, P. aeruginosa* and *K. pneumoniae*) exhibited comparatively lower sensitivity. The standard antibiotic, streptomycin, produced consistent inhibition zones of 9-11 mm across all organisms, serving as a positive control.

The present study clearly demonstrated that the antimicrobial activity of *Rothia indica* leaf extracts varied depending on the solvent used, with the ethanol extract showing the most significant inhibitory effect against all tested pathogens. This can be attributed to the fact that ethanol is a polar solvent capable of extracting a wide range of bioactive compounds, including phenolics, flavonoids, alkaloids and tannins, which are known for their antimicrobial properties[25]. These compounds disrupt microbial cell walls, interfere with protein synthesis and inhibit the activity of enzymes. The ethanol extract demonstrated notable broad-spectrum activity against *Escherichia coli*, *Pseudomonas aeruginosa and Klebsiella pneumoniae*, producing inhibition zones approaching those of streptomycin (9-11 mm in diameter). However, it is important to note that while some zones are comparable, plant extracts are crude mixtures, whereas streptomycin is a pure and highly potent antibiotic compound. Therefore, the presence of similar zones suggests the presence of strong bioactive constituents in the extract.

The moderate activity observed in the ethyl acetate extract further supports the presence of semi-polar antimicrobial compounds. In contrast, the poor activity of the petroleum ether extract indicates that non-polar compounds contribute less to antimicrobial activity, which aligns with earlier findings[27]. The aqueous extract exhibited selective activity, particularly against *Staphylococcus aureus*, potentially due to the presence of water-soluble bioactive compounds such as glycosides and phenolics. The antimicrobial activity of *Rothia indica* leaf extracts showed comparable inhibition zones to the standard antibiotic streptomycin in certain cases, particularly against *Staphylococcus aureus*, where the aqueous extract exhibited a maximum zone of inhibition (11 mm), similar to that of the standard drug. In contrast, certain extracts (particularly petroleum ether) showed minimal or no activity, indicating that the active antimicrobial compounds are likely polar or semi-polar in nature, which is consistent with previous studies[27].

Cytotoxicity: The brine shrimp lethality assay (BSLA) is a simple and inexpensive bioassay for evaluating the potency of phytochemicals in plant extracts. The results of this study showed a direct relationship between the extract concentration and degree of lethality. Following a 24 hrs observation period, all the shrimp in the control group survived. Nevertheless, the highest mortality was noted at 1000 ppm and the lowest at 1 ppm.

Cytotoxicity was studied for all four leaf extracts listed in Table 3. Three dilutions were considered replicates. The %mortality was based on counting the number of motile nauplii and subtracting it from the total nauplii to obtain the number of dead organisms and the percentage was calculated. The LC_{50} value of the ethanolic extract was the highest among all the extracts (LC_{50} -1.38), followed by ethyl acetate, aqueous and Petroleum ether extract (LC_{50}-2.56, 3.71 and 17.39, respectively). The negative control (Tween 80) showed no mortality (0%), confirming that the experimental conditions were not toxic. The positive control exhibited high mortality, ranging from 86.7% to 96.7%, thereby validating the assay system

The highest larval mortality was recorded at a concentration of 1000 ppm of ethanol extract (93.4%) and as the concentration decreased (1 ppm), the mortality percentage also declined, followed by those of ethyl acetate and aqueous extracts. This suggests that polar solvents are more effective in extracting biologically active compounds, such as flavonoids, phenolics and alkaloids, which are known to exhibit cytotoxic and pharmacological properties[28,29]. The relatively low activity of the pleum ether extract may be attributed to differences in the composition and concentration of bioactive constituents extracted by the respective solvents[30].

The observed dose-dependent increase in mortality confirms that the toxicity of the extracts is concentration-dependent, which is a characteristic feature of biologically active compounds. According to the brine shrimp lethality assay, LC_{50} values below 1000 ppm are generally considered indicative of bioactivity[5]. In the present study, all extracts exhibited LC_{50} values well below 1000 ppm, indicating significant bioactivity, with the ethanol extract showing particularly potent effects. This suggests the presence of strong cytotoxic constituents, which may be further explored for potential pharmacological applications. The results are in agreement with recent studies reporting that plant-derived extracts rich in secondary metabolites exhibit toxicity against brine shrimp nauplii and can serve as a preliminary indicator of pharmacological activity[16,31].

CONCLUSION

The present study demonstrated that *Rothia indica* leaf extracts possess significant phytochemical constituents and notable biological activities. The presence of bioactive compounds, such as alkaloids, flavonoids, tannins and phenolics, was confirmed through preliminary phytochemical screening. Among the different solvent extracts, the ethanol extract exhibited the highest antimicrobial activity against the tested bacterial pathogens, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, showing effects comparable to those of the standard antibiotic in certain cases. Cytotoxicity analysis using the *Artemia salina* lethality assay revealed a dose-dependent increase in mortality, with the ethanol extract exhibiting the lowest LC_{50} value, indicating strong cytotoxic potential. Collectively, these findings suggest that *Rothia indica* is a promising source of bioactive compounds with potential applications in the antimicrobial and pharmacological fields. This study provides preliminary scientific evidence supporting its medicinal value and further investigations are required for the isolation and characterization of active compounds.

SIGNIFICANCE STATEMENT

The present study highlights the therapeutic potential of Rothia indica leaves by demonstrating their phytochemical richness, antibacterial activity and cytotoxic effects. The findings identify the ethanol extract as particularly promising, suggesting that Rothia indica may serve as a valuable source of bioactive compounds for further pharmacological investigation and development of plant-based therapeutic agents.

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