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Antimicrobial Screening of Plant-derived extracts against drug resistant bacterial Strains isolated from Diabetic Patients: An *In vitro* Study

[1*]Manisha Jain, [2]Arvind Kumar Shakya

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Corresponding Author Email: manisha.jain@universal.edu.in

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ABSTRACT:

Background: The emergence of drug-resistant organisms poses a significant challenge to world health. Infections associated with diabetic patients exemplify this phenomenon, as the prevalence of multidrug-resistant organisms is rising in these infections. Consequently, it is imperative to identify novel sources of anti-infective agents that could offer safe and more cost-effective alternatives to current medications. Therefore, the current work sought to perform an antibacterial evaluation of some crude plant extracts against bacterial strains obtained from diabetic patients. **Materials and methods**: Antibacterial susceptibility of the bacteria isolated from diabetic patients was determined by Kirby-Bauer disc diffusion method. The antibacterial screening of the methanolic extracts obtained from 28 plants, was performed using Agar-well diffusion method. The Minimum Inhibitory Concentration (MIC) of methanolic extracts of *Rosa centifolia* and *Punica granatum* showing strongest activity were determined by broth dilution method. **Results:** Antibacterial susceptibility testing revealed *Staphylococcus aureus* and *Enterobacter spp.* as most susceptible organism, whereas *Klebsiella spp.* exhibited the greatest resistance, followed by *Escherichia coli, Pseudomonas aeruginosa and Streptococcus spp.* Plant extracts of *Rosa centifolia* (Rose) and *Punica granatum* (Pomegranate) exhibited the strongest antimicrobial activity with a highest inhibition zone of 22.3±1 mm and 22.3±0.57 mm respectively against *Staphylococcus aureus. Escherichia coli, Klebsiella spp.* and *Enterobacter spp.* found completely resistant. Pomegranate and rose found more effective in MIC determination. MIC of pomegranate was determined as 0.5mg/ml against all the tested organisms. **Conclusion:** Methanolic extracts of Rose and pomegranate showed the highest antibacterial efficacy, possibly due the abundance of antimicrobial phenolic components in the extracts. However, this study provides an *in vitro* analysis of crude plant extracts, a further detailed Phyto-toxicological analysis can determine the active principle and *in vivo* toxicity of these plant extracts and they can potentially be employed in alternative or complementary therapies for diabetes-related infections.

KEYWORDS: Antimicrobial screening, diabetes, drug resistance, infections, in-vitro study, plant extracts..

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1. INTRODUCTION

Diabetes mellitus is one of the most common metabolic disorders predisposing for infectious diseases. Diabetic individuals are more susceptible to bacterial infections, with a rising prevalence of multidrug-resistant pathogens. The microbial resistant against antibiotic drugs makes it very hard to find efficient antimicrobial medications to fight against bacterial infection. Because people with diabetes often need antibiotics to treat infections, it's crucial to look at alternative treatments that have natural antibacterial characteristics. Indian traditional medical system like Ayurveda has been using plant-based medicines for a long time to cure different bacterial illnesses.

According to the World Health Organization (WHO) about 80% of the people worldwide use traditional or folk medicine in many forms for the treatment of their primary healthcare. The secondary metabolites such as phenolic and flavonoids present in medicinal plants showed many pharmacological properties including antimicrobial effects. [1, 2] Throughout history, plant-derived essential oils and extracts have garnered interest as natural remedies for numerous infectious diseases. [3] As the increase in antimicrobial resistance among microorganisms limits antibiotic choices, the herbal medicine strategies have become more relevant. [4] In response to growing drug resistance, researchers are increasingly focusing on natural sources to develop complementary and alternative treatment options. [5] Phytochemicals and plant extracts have a promising role in therapeutic applications against multidrug resistant bacteria. [6]

Despite the extensive use of plant-based treatments in traditional medicine, there remains a scarcity of scientific data on the antibacterial effects of many selected plants against urinary tract pathogens and bacteria causing foot infections in diabetic patients. [7] This underscores the urgent need to identify novel sources of anti-infective agents that offer safe and cost-effective alternatives to current medications. Some of the plants examined in our study have been identified in traditional literature to possess medicinal and antimicrobial attributes. This study aims to promote a more informed application of these plant materials in the development of new antibacterial agents. To achieve this, systematic and methodical investigations have been designed to assess the potential antimicrobial activities of various plant derived extracts against a range of test bacteria.

2. MATERIALS AND METHODS:

2.1 Equipment, media and Reagents

The octodisks were procured from Himedia, Mumbai for gram negative (G-III-minus) and gram positive bacteria (combi VII). Soxhlet apparatus used for the extraction of plants. Agar, methanol and other chemicals were used of analytical grade in this study.

2.2 Sample isolates: The microbial isolates utilized in this investigation were sourced from routine clinical samples (diabetic patients) processed at the Diagnostic Centre in Gwalior (M.P.), as part of conventional diagnostic and treatment protocols. No further samples were acquired explicitly for research reasons**.** Antibacterial evaluations of plant extracts against bacterial strains were performed at the Department of Microbiology, College of Life Sciences (Jiwaji University, Gwalior M.P.) India. The randomization and blinding were not applicable since the research was an *In vitro* laboratory-based antibacterial test without subjective outcome evaluation.

The bacteria were isolated from the samples of urinary tract infections (UTI) and foot infections obtained from diabetic patients. For this study, we included bacterial isolates that were clinically relevant, properly identified, and confirmed as pure cultures using standard microbiological methods. We excluded duplicate samples from the same patient, mixed or contaminated cultures, poorly identified strains, non-viable organisms, and any isolates not obtained from clinical sources. Multiple organisms from one specimen were acceptable, but only the first isolate of a particular species per patient for the entire collection period was accepted. Duplicate isolates (same genus and species) obtained from the same or different specimens were excluded.

2.3 Antibacterial Susceptibility Test

In the present investigation, antibacterial susceptibility of test bacteria isolated from diabetic patients was performed by using octodisks. The gram positive isolates included in the study are *Staphylococcus aureus and Streptococcus spp., Escherichia coli, Klebsiella spp., Enterobacter spp.,* and *Pseudomonas aeruginosa are among gram negative isolates*. Octodisks for gram negative bacteria contained Amikacin (10µg), Carbenicillin (100µg) Ciprofloxacin (10µg), Co-trimazine (25 µg), Kanamycin (30µg), Tetracycline (30µg), Nitrofurantoin (300µg) and Streptomycin (10µg) and octodisks for gram positive bacteria contained Amoxicillin (10µg), Cloxacillin (5µg), Erythromycin (15µg), Tetracycline (10µg), Penicillin (2 units), Co-trimoxazole (25µg), Penicillin V(3µg), Cephalexin (30 µg). Kirby-Bauer disc diffusion method was used to determine antibiotic susceptibility using Mueller Hinton agar. [8] Few well isolated colonies of test bacteria were picked from the culture plates and inoculated into the Mueller-Hinton broth. Broth was incubated for 3-6 hours at 37^0 C, to attain a turbidity of 0.5 McFarland. A sterile cotton swab was dipped into the standardized suspension having 1.5 x 10^8 CFU (colony forming units) /ml. The swab was drained and inoculated on a Mueller-Hinton Agar (MHA) plate by spreading evenly on the entire surface. Subsequently antibacterial octodisks were mounted onto the inoculated plates and pressed to ensure proper contact and incubated at 35 ± 2 °C for 16–18 hours. The diameter of the zone of inhibition was measured to the nearest in millimeter after the incubation and calculated as the mean of three replicates.

2.4 Antibacterial Activity of plant extracts

The antibacterial screening of leaf extracts from 28 plant species (Table1) was performed against six selected bacterial strains. The inclusion criteria of plants examined in the study was based on ethno botanical relevance, local availability, and literature-documented antimicrobial activity.

2.4.1 Collection and Preparation of extracts

Fresh leaves from all plant species selected for this study were locally sourced, identified, and authenticated by the Department of Botany, Jiwaji University, Gwalior M.P. (India). The leaves were thoroughly washed 2-3 times with distilled water and methanol and then shade-dried until completely dehydrated. Upon drying, the leaves were ground into a fine powder which was steeped in methanol for one night before extraction by placing it in a cellulose thimble. Plant powder (25 g) was dissolved in 250 ml of methanol for 8 hours for extraction in Soxhlet apparatus. The crude extracts were allowed to dry at room temperature and then transferred in a sealed container to store at 4°C until further analysis.

2.4.2 Formulation of extracts

For the antibacterial activity study, 500 mg of dried methanolic extract from each plant was reconstituted in 0.5% (v/v) of 1 ml methanol to prepare 500 mg/ml solution.

Table 1: List of selected plants used in the study

Sr. No.	Common name	Botanical name	Family
1	Neem	*Azadirachta indica*	Meliaceae
2	Bougainvillea	*Bougainvillea glabra*	Nyctaginaceae
3	Jamun	*Eugenia jambolana*	Myrtaceae
4	Guava	*Psidium guajava*	Myrtaceae
5	Champa	*Michelia champaca*	Magnoliaceae
6	Gudmar	*Gymnema sylvestre*	Apocynaceae
7	Custard Apple	*Annona squamosa*	Annonaceae
8	Mango	*Mangifera indica*	Anacardiaceae
9	Rose	*Rosa centifolia*	Rosaceae
10	Thuja	*Thuja orientalis*	Cupressaceae
11	Ashok	*Saraca Indica*	Fabaceae

12	Tulasi	*Ocimum sanctum*	Labiateae
13	Marigold	*Calendula officinalis*	Asteraceae
14	Lemon	*Citrus limon*	Rutaceae
15	Sewanti	*Chrysanthemum coronarium*	Asteraceae
16	Elephant ear (Taro)	*Colocasia esculenta*	Araceae
17	Siris	*Albizia lebbeck*	Fabaceae
18	Aak	*Calotropis procera*	Asclepiadaceae
19	Datura	*Datura stramonium*	Solanaceae
20	Belpatra	*Aegle marmelos*	Rutaceae
21	Gudhal	*Hibiscus rosa sinensis*	Malvaceae
22	Gulmohar	*Delonix regia*	Fabaceae
23	Christmas tree	*Araucaria heterophylla*	Araucariaceae
24	Yellow oleander	*Cerbera thevetia*	Apocynaceae
25	Sadabahar	*Vinca rosea*	Apocynaceae
26	Pomegranate	*Punica granatum*	Punicaceae
27	Chameli	*Jasminum grandiflorum*	Oleaceae
28	Ground nut	*Arachis hypogaea*	Fabaceae

2.4.3 Preparation of inoculum

The bacterial isolates were inoculated into Mueller Hinton Broth medium and incubated for3-6 hours at 37^{o}C to attain turbidity about 0.5 McFarland standards. The final inoculum size was adjusted at concentration 1.5 x 10^{8}CFU/ml Following CLSI guidelines. [9]

2.4.4 Antimicrobial Susceptibility Test

Susceptibility tests were conducted using the agar-well diffusion method. [10] The antimicrobial activity of the plant extracts was assessed using the Muller Hinton Agar. 1 ml of the standard bacterial suspension was spread evenly over the entire surface of Mueller Hinton Agar plates using a sterile swab and the plates were dried at room temperature. After drying, wells (6-mm) were aseptically prepared in the agar using a cork borer, with four wells punched in each plate. Prior to processing, each plant extract was vortexed. A 40µl volume of each extract (500 mg/ml, reconstituted in methanol) was added to the wells (20 mg per well).

The plates were then incubated at 37^{0}C for 24 hours. Measurement of the Inhibition Zone Diameter (IZD) was carried out using the Hi Antibiotic Zone scale (Himedia) to the nearest millimeter (mm). Chloramphenicol was used as a positive control. The stock solution of Chloramphenicol was prepared to get the concentration of 30 µg / well equivalent to the standard inhibitory concentration of the antibiotic. All the tests were carried out in triplicate. MICs of most active extracts were also determined.

2.5 Determination of minimal inhibitory concentration (MIC)

Broth dilution assay method with slight modification was used to measure the MICs of Methanolic extracts of *Rosa centifolia* and *Punica granatum*. [10] In tubes containing 1 ml of sterile Mueller Hinton Broth, methanolic extracts were serially diluted in the concentration range of 0.5-4 mg/ml by using Two-fold serial dilutions. 1 ml of test culture suspension having standard concentration of 5 x 10^{5} CFU/ml was seeded into each dilution and control tubes. The positive control tube contained the test bacteria without extract and the negative control tube had extract only with no test organism added into it. Incubation at 37^{0}C for 24 hours was carried out. The MIC was taken as the lowest concentration of the extract that did not show any visible growth.

3. RESULTS:

3.1 Sample isolates:

A total of 200 bacteria were isolated from 150 urine samples while 114 bacteria isolated from 50 samples of foot infections of diabetic patients (Table 2). *Pseudomonas aeruginosa, S. aureus* and *Streptococcus spp.* isolated from diabetic foot infection and *E. coli, Klebsiella spp., Enterobacter spp*. isolated from urine samples of diabetic patients were selected for the antibacterial study.

Table 2: Distribution of Bacteria Isolated from Diabetic Foot Lesions and Urinary Tract Infections

Bacterial Isolates	Diabetic Foot Lesions (n=114)	Percentage (%)	Urinary Tract Infections (n=200)	Percentage (%)
Klebsiella spp.	25	22	20	10
Pseudomonas aeruginosa	35	31	23	11.5
Staphylococcus aureus	44	38	30	15
Streptococcus spp.	10	9	7	3.5
Escherichia coli	10	–	112	56
Enterobacter spp.	–	–	6	3
Total	114	100	200	100

3.2 Antibacterial susceptibility test:

Antibacterial susceptibility results obtained by the disc diffusion method are summarized in Table 3 & Table 4 and displayed in Fig.1 & 2 for gram positive and gram-negative bacteria, respectively. There was a significant difference observed in the antibiogram of isolated bacteria. All the gram-negative bacteria were susceptible against amikacin and were resistant to carbenicillin and tetracycline. *E. coli* and *Pseudomonas aeruginosa* were resistant to 62.5 % of antibacterial drugs tested. *Enterobacter spp.* was the most susceptible organism among all. *Klebsiella spp*. exhibited a high frequency of resistance to the antibacterials tested and found only susceptible to amikacin. Between gram positive bacteria, *S. aureus* was found susceptible to all the antibacterial agents except amoxycillin and 50% antibacterial drugs used in the study were found to be effective against *Streptococcus spp.* Both the organisms were highly susceptible to co-Trimoxazole and Penicillin.

Table 3: Antibacterial susceptibility of gram positive bacteria

S. No.	Antibacterial drug	Symbol	Concentration	Zone of inhibition (mm)* against bacteria	
				Staphylococcus aureus	***Streptococcus spp.***
1	Amoxycillin	Am	10µg	Res	Res
2	Cloxacillin	Cx	5µg	19.3±.58	Res
3	Erythromycin	E	15µg	26.3±.58	Res
4	Co-Trimoxazole	Co	25µg	26.6±1	28.3±.58
5	Penicillin	P	2 units	36.3±.58	26.3±.58
6	Tetracycline	T	10µg	24±1	Res
7	Penicillin V	PV	3 µg	28.3±.58	20.6±1
8	Cephalexin	Cp	30µg	20±1	20.3±.58
Res = resistant, * =mean of three replicates± standard deviation					

Table 4: Antibacterial susceptibility of gram negative bacteria

S. No.	Antibacterial drug	Symbol	Concentration	Zone of inhibition (mm)* against bacteria			
				E. coli	***Klebsiella spp.***	***Enterobacter spp.***	***Pseudomonas aeruginosa***
1	Amikacin	Ak	10µg	19.3±.58	18±1	20.6±1	19.3±.58
2	Carbenicillin	Cb	100µg	Res	Res	Res	Res

3	Ciprofloxacin	Cf	10µg	Res	Res	25.3±.58	33.3±.58
4	Co-Trimazine	Cm	25µg	Res	Res	27±1	Res
5	Kanamycin	K	30µg	Res	Res	20.3±.58	Res
6	Tetracycline	T	30µg	Res	Res	Res	Res
7	Nitrofurantoin	Nf	300µg	20.6±1	Res	Res	Res
8	Streptomycin	S	10µg	17±1	Res	16.3±.58	18.3±.58

Res = resistant, * =mean of three replicates± standard deviation

Fig. 1: Antimicrobial Activity of drugs against gram positive bacteria: amoxicillin; Am (10µg), cloxacillin; Cx (5µg), erythromycin; E (15µg), tetracycline; T (10µg), penicillin; P (2 units), co-trimoxazole; Co (25µg), penicillin v; Pv (3µg), cephalexin ;Cp (30 µg).

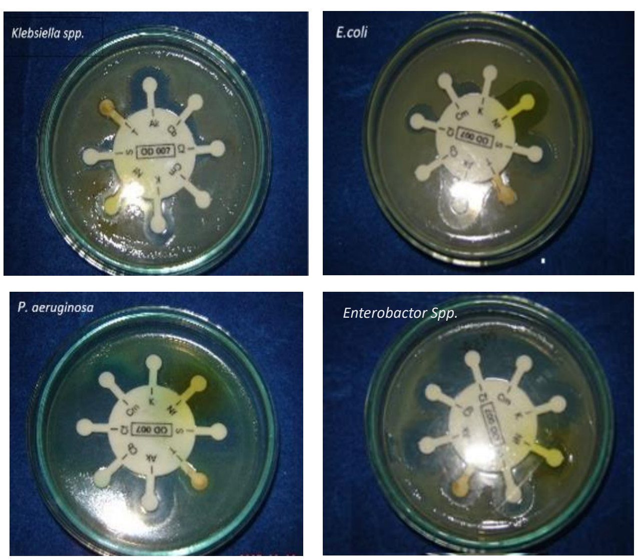

Fig. 2: Antimicrobial Activity of drugs against gram negative bacteria; amikacin; Ak (10µg), carbenicillin; Cb (100µg) ciprofloxacin; Cf (10µg), co-trimazine; Cm (25 µg), kanamycin; K (30µg), tetracycline; T (30µg), nitrofurantoin; Nf (300µg) and streptomycin; S (10µg)

3.3 Extraction of Plants:

The extraction of 25 g of dried leaves with methanol produced plant extract residues between the range of 2.01g to 11.524 g. *Punica granatum* (11.524g) gave the highest yield, followed by *Datura stramonium* (7.843 g) and *Rosa centifolia (7.846 g)* while *Psidium guajava* (2.01g) gave the lowest extract yield. Table 5 presents the total yield obtained after extraction.

Table 5: Extract obtained from 25 g* leaves after Soxhlet extraction

Sr. No	Name of Plant	Total yield in grams after extraction
1	*Azadirachta indica*	4.59
2	*Bougainvillea glabra*	4.97
3	*Eugenia jambolana*	3.865
4	*Psidium guajava*	2.01
5	*Michelia champaca*	2.4
6	*Gymnema sylvestre*	4.208
7	*Annona squamosa*	4.11
8	*Mangifera indica*	6.479
9	*Rosa centifolia*	7.486
10	*Thuja orientalis*	3.882
11	*Saraca Indica*	6.825
12	*Ocimum sanctum*	3.99
13	*Calendula officinalis*	3.319
14	*Citrus limon*	4.818
15	*Chrysanthemum coronarium*	6.313
16	*Colocasia esculenta*	3.319
17	*Albizia lebbeck*	6.269
18	*Calotropis procera*	3.102
19	*Datura stramonium*	7.843

20	*Aegle marmelos*	2.607
21	*Hibiscus rosa sinensis*	3.04
22	*Delonix regia*	3.02
23	*Araucaria heterophylla*	4.563
24	*Cerbera thevetia*	4.288
25	*Vinca rosea*	4.365
26	*Punica granatum*	11.524
27	*Jasminum grandiflorum*	3.02
28	*Arachis hypogaea*	3.132

3.4 Antimicrobial Susceptibility of plant extracts

Table 6 presents the antibacterial activities of methanolic extracts from 28 plant species against selected bacterial isolates. The statistical analysis in this study was conducted using SPSS 27 to evaluate the antibacterial activity of 28 plant extracts against *Staphylococcus aureus*, *Streptococcus spp.*, and *Pseudomonas aeruginosa* only, the other bacteria were omitted as they found completely resistant to the extracts. The inhibition zones, measured in millimetres, were recorded in triplicate for each extract, and average values were computed to minimize experimental error. Descriptive statistics including mean, range, standard deviation, and variance were first calculated to provide a baseline understanding of the distribution and variability of antibacterial activity across the three bacterial species. To determine the suitability of parametric tests, the Kolmogorov–Smirnov and Shapiro–Wilk tests were applied to assess normality. Since all p-values were below the 0.05 threshold, the data deviated significantly from normality, thereby justifying the use of non-parametric statistical methods.

For overall comparison of inhibition zones among the three bacterial species, the Friedman test was employed, which is appropriate for related samples under non-parametric conditions. The test yielded a highly significant chi-square statistic, confirming differences in susceptibility among the species. To further identify the specific pairs of bacterial species that differed significantly, the Wilcoxon Signed-Rank Test was applied for pairwise comparisons. Given that multiple comparisons increase the risk of Type I error, the Bonferroni correction was used to adjust the significance threshold. With three comparisons, the adjusted α was set at 0.0033, ensuring stricter control of false positives. The Kruskal–Wallis test was conducted to compare the antibacterial activity of 28 plant extracts against *Staphylococcus aureus*, *Streptococcus* species, and *Pseudomonas aeruginosa*.

Table 6: Antibacterial activity of plant extracts against selected bacterial isolates

Sr. No.	**Plant extracts**	**Zone size in mm ***			**Sr. No.**	**Plant extracts**	**Zone size in mm***		
		S. aureus	***Streptococcus spp.***	***P.aeruginosa***			***S. aureus***	***Streptococcus spp.***	***P.aeruginosa***
1	*Azadirachta indica*	1.3333	1.6667	0.0000	15	*Chrysanthemum coronarium*	13.3333	0.0000	0.0000
2	*Bougainvillea glabra*	1.6667	0.0000	0.0000	16	*Colocasia esculenta*	0.0000	11.3333	0.0000
3	*Eugenia jambolana*	13.6667	13.6667	12.6667	17	*Albizia lebbek*	11.0000	0.0000	10.6667
4	*Psidium guajava*	2.3333	11.6667	0.0000	18	*Calotropis procera*	0.0000	0.0000	0.0000
5	*Michelia champaca*	15.0000	0.0000	0.0000	19	*Datura stramonium*	12.6667	13.6667	0.0000

6	*Gymnema sylvestris*	1.3333	0.0000	2.0000	20	*Aegle marmelos*	0.0000	0.0000	0.0000
7	*Annona squamosa*	4.3333	0.0000	0.0000	21	*Hibiscus rosa sinensis*	1.3333	0.0000	2.6667
8	*Mangifera indica*	16.0000	16.3333	5.3333	22	*Delonix regia*	0.0000	0.0000	0.0000
9	*Rosa centifolia*	22.3333	17.3333	15.3333	23	*Araucaria heterophylla*	11.6667	0.0000	0.0000
10	*Thuja orientalis*	13.6667	12.3333	0.0000	24	*Cerbera thevetia*	0.0000	0.0000	0.0000
11	*Saraca Indica*	13.6667	13.3333	0.0000	25	*Vinca rosea*	22.0000	7.3333	0.0000
12	*Ocimum sanctum*	25.3333	0.0000	7.6667	26	*Punica granatum*	22.3333	18.0000	13.0000
13	*Calendula officinalis L*	15.6667	0.0000	2.6667	27	*Jasminum grandiflorum*	20.6667	0.0000	14.6667
14	*Citrus limon*	11.3333	0.0000	2.6667	28	*Arachis hypogea*	0.0000	0.0000	0.0000

***** *=Average Zone of Inhibition(Mean of three replicates)*

Each plant extract showed a significant difference in the inhibitory effect against the isolates (Table 6). Nineteen plant species shown effectiveness against at least one isolate of bacteria out of which *Rosa centifolia* (rose) and *Punica granatum* (pomegranate) extracts were significant for their potent antibacterial properties against *Streptococcus* species, *Pseudomonas aeruginosa*, and *Staphylococcus aureus.* Notable efficacy showed by *E. jambolana* against these isolates as well. Several extracts, such as *Ocimum sanctum*, *Rosa centifolia*, *Punica granatum,* and *Vinca rosea*, exhibited comparatively larger inhibition zones, particularly against *S. aureus*. The greatest inhibition zone was found to be 25.3±0.57 mm with *Ocimum sanctum* against *Staphylococcus aureus*, indicating its antibacterial activity only against this bacterium.

The descriptive statistics of the average inhibition zones (mm) of 28 plant extracts tested against three bacterial species (*S. aureus, Streptococcus spp.,* and *P. aeruginosa*). The values represent the mean of triplicate assays for each extract.

Among the three species, *S. aureus* showed the widest range of inhibition zones (25.33 mm) with a mean of 9.74 mm, indicating greater variability in susceptibility across the extracts (SD = 8.47, variance = 71.78). *Streptococcus spp*. exhibited a narrower range (18.00 mm) and a lower mean inhibition zone (4.88 mm), with moderate variability (SD = 6.80). *P. aeruginosa* was the least affected, with the smallest mean inhibition zone (3.19 mm) and lowest variance (26.70), reflecting limited antibacterial activity of the extracts against this species.

These descriptive results provide a baseline understanding of the distribution and variability of antibacterial activity, and they form the foundation for subsequent statistical analyses (normality tests, Friedman test, and Wilcoxon pairwise comparisons).

Kolmogorov–Smirnov and Shapiro–Wilk tests conducted to assess the normality of the averaged inhibition zone data for the three bacterial species (*S. aureus, Streptococcus spp., and P. aeruginosa*).

For *S. aureus*, the Kolmogorov–Smirnov statistic was 0.202 ($p = 0.005$) and the Shapiro–Wilk statistic was 0.878 ($p = 0.004$). For *Streptococcus spp.*, both tests yielded highly significant results (Kolmogorov–Smirnov = 0.371, $p < 0.001$; Shapiro–Wilk = 0.700, $p < 0.001$). Similarly, *P. aeruginosa* showed significant deviations from normality (Kolmogorov–Smirnov = 0.339, $p < 0.001$; Shapiro–Wilk = 0.664, $p < 0.001$).

Since all p-values were below the 0.05 threshold, the data for each bacterial species did not follow a normal distribution. This justified the use of non-parametric statistical tests (Friedman test for overall differences and Wilcoxon Signed Rank test for pairwise comparisons) in the subsequent analysis.

The Friedman test conducted to compare the antibacterial activity of 28 plant extracts against *S. aureus*, *Streptococcus spp.*, and *P. aeruginosa*. The test yielded a chi square value of 17.035 with 2 degrees of freedom (N = 28), and the result was statistically significant ($p < 0.001$) Since the obtained p-value was far below the 0.05 threshold, the differences are significant at the 95% confidence level. Moreover, because $p < 0.001$, the findings remain significant even at stricter thresholds, including the 99% and 99.9% confidence levels, indicating robust evidence against the null hypothesis.

This confirms that there were significant differences in the inhibition zones among the three bacterial species. Specifically, the susceptibility of the bacteria to the plant extracts was not uniform. These findings justify further pairwise comparisons using the Wilcoxon Signed Rank Test to identify which bacterial species differed significantly from each other.

The Wilcoxon Signed-Rank Test was used for the pairwise comparisons of antibacterial activity among the three bacterial species. The comparison between *S. aureus* and *Streptococcus spp.* yielded a Z-value of –3.004 with p = 0.003, indicating a statistically significant difference at the 99% confidence level. Similarly, the comparison between *S. aureus* and *P. aeruginosa* was highly significant (Z = –3.880, $p < 0.001$), confirming significance at the 99% confidence level and remaining robust even under stricter thresholds (99.9%). In contrast, the difference between *Streptococcus spp.* and *P. aeruginosa* was not statistically significant (Z = –1.089, p = 0.276), indicating no difference at the 99% confidence level.

To control for Type I error across multiple pairwise comparisons, the Bonferroni correction was applied. With three comparisons, the adjusted threshold was $\alpha = 0.0033$. Under this stricter criterion:

- *S. aureus* vs *Streptococcus spp.* (p = 0.003) remained significant.
- *S. aureus* vs *P. aeruginosa* ($p < 0.001$) was highly significant.
- *Streptococcus spp.* vs *P. aeruginosa* (p = 0.276) was not significant.

These findings confirm that *S. aureus* was significantly more susceptible to the plant extracts compared to both *Streptococcus spp.* and *P. aeruginosa*. However, no meaningful difference was observed between *Streptococcus spp.* and *P. aeruginosa*, suggesting similar levels of resistance to the tested extracts.

Table 7: The Kruskal–Wallis test for comparative antibacterial activity of plant extracts

Bacterial isolates	χ^2	df	p-value	ε^2 (Effect size)
Staphylococcus aureus	82.3	27	< 0.001	0.991
Streptococcus spp.	82.8	27	< 0.001	0.997
Pseudomonas aeruginosa	82.8	27	< 0.001	0.998

The Kruskal–Wallis test (Table 7) analysis revealed highly significant differences among the plant groups for all three bacteria (*S. aureus*: $\chi^2 = 82.288$, df = 27, $p < 0.001$; *Streptococcus* species: $\chi^2 = 82.788$, df = 27, $p < 0.001$; *P. aeruginosa*: $\chi^2 = 82.833$, df = 27, $p < 0.001$). The effect size (ε^2) values were very high (0.991–0.998), indicating a strong effect of the treatments on antibacterial activity. These findings indicate that the inhibitory effects of the plant extracts were not uniform, and certain plants demonstrated markedly greater antibacterial activity than others.

The descriptive analysis of inhibition zones was done to highlight clear differences in antibacterial effectiveness among the plant extracts. Against *Staphylococcus aureus*, *Ocimum sanctum* (median 25 mm) was the most potent, followed closely by Plants *Rosa centifolia, Punica granatum*, and *Vinca rosea*, each showing median zones above 21 mm. For *Streptococcus* species, *Punica granatum* (median 18 mm) exhibited the strongest activity, with Plants *Rosa centifolia, Mangifera indica*, and *Eugenia jambolana* also demonstrating notable inhibition ranging from 14–17 mm. In the case of *Pseudomonas aeruginosa*, which is generally more resistant, Plant *Rosa centifolia* (median 15 mm) was the most effective, while Plants *Jasminum grandiflorum, Eugenia jambolana,* and *Punica granatum* showed moderate activity with median zones between 12–13 mm. These findings confirm that *Rosa centifolia* and *Punica granatum* plant extracts possess significant antibacterial properties, underscoring their potential for targeted therapeutic applications. In contrast, a number of extracts showed little or no measurable activity across the tested species. However, none of the plant extracts, including those from Rose and Pomegranate, showed activity against *Escherichia coli*, *Klebsiella spp.* and *Enterobacter spp.*

3.4 Minimum Inhibitory Concentration (MIC)

The methanolic extracts of *Rosa centifolia* and *Punica granatum* showed minimum inhibitory concentration (MIC) ranged from 0.5 to 4 mg/ml against the bacterial isolates, which showed the strongest activity among the other extracts (Table 8). In contrast to the agar assay, both plants extracts demonstrated inhibitory effects on both gram-negative and gram-positive bacteria. When compared to *Rosa centifolia, Punica granatum* showed a stronger antimicrobial ability to suppress the growth cycle of gram-negative bacteria. The MIC of *Punica granatum* was 0.5 mg/ml for all the tested bacteria. For *Rosa centifolia*, the MIC was also 0.5 mg/ml for all bacteria except *Pseudomonas aeruginosa* and *Klebsiella* spp., for which the MIC was >4 mg/ml.

Table 8: MIC* of selected plant extracts (mg/ml)† against test bacteria

Plant extracts	*S. aureus*	*Streptococcus spp.*	*E.coli*	*Klebsiella spp.*	*Enterobacter spp.*	*P. aeruginosa*
Rosa centifolia	0.5±0	0.5±0	0.5±0	>4	0.5±0	>4
Punica granatum	0.5±0	0.5±0	0.5±0	0.5±0	0.5±0	0.5±0

* = Minimum Inhibitory Concentration

† = Mean of three replicates± standard deviation

4. DISCUSSION:

The results of present study revealed that while *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common pathogens in diabetic foot lesions, *Escherichia coli* remains the predominant pathogen associated with urinary tract infections.

This study investigated the antibacterial susceptibility pattern of bacterial isolates from the diabetic patients. Some of the antimicrobial antibiotics and chemotherapeutic agents tested in the present study found to be inhibitory for the test bacteria (Table 3 & 4). Among the urinary tract pathogens (Fig.2), including *Klebsiella* spp., *Enterobacter* spp., and *E. coli*, resistance to tetracycline and carbenicillin was observed. It was found that *Klebsiella spp.* was resistant against all the antibacterial agents tested and it only showed sensitivity to amikacin. While *E. coli* is more vulnerable to nitrofurantoin, a medication frequently used to treat urinary tract infections, *Enterobacter spp.,* the most susceptible of the three species, demonstrated resistance to it. *E. coli,* however, showed resistance to 62.5% against tested antibacterial drugs. Among all the antibacterial drugs tested, amikacin proved to be the most effective against all the Gram-negative bacteria aligning with the similar findings. [11] Co-trimoxazole and penicillin showed the greatest efficacy against both Gram-positive strains (Fig.1). *Staphylococcus aureus* and *Enterobacter* spp. were the most susceptible organisms, while *the* most resistant bacteria were *Klebsiella species, Escherichia coli,* and *Pseudomonas aeruginosa* respectively. Unlike Gram-positive bacteria, treating Gram-negative bacteria with antibiotics like β-lactams and quinolones is often more challenging due to their outer membrane, which serves as a barrier to hinder their specific cellular targets. [12]

As the number of bacteria that are resistant to antibiotics rises, plant medicines are considered to have antibacterial phytocompounds for the prevention of pathogenic bacterial infection. In this research, methanolic extract from 28 different plants were tested and found to demonstrate effective antimicrobial activity against different types of bacteria, especially *Rosa centifolia* and *Punica granatum* exhibited potential inhibitory action against growth of Gram-positive bacteria.

Several methods of extraction have been used by the researchers for greater yield and efficacy. Methanol being a highly polar solvent has been used for the extraction of a wide range of phytochemicals.[13] A relatively higher concentration of extracts were used in the wells due to the crude nature of extracts containing complex mixture of active compounds. Higher loading of extracts ensured the proper diffusion of bioactive compound into the medium.

Our findings indicate that plant methanolic extracts possess significant antibacterial activity. Antimicrobial activity of methanolic extracts can be attributed to the presence of several phenolic compounds. [14, 15] Methanolic extracts have been found to be effective in diabetic wound healing due to the antioxidant properties. [16]

The percentage yield obtained from methanolic leaf extracts of *Punica granatum* was highest among all the extracts, followed by *Datura stramonium* and *Rosa centifolia* (Table 5). Presence of an appreciable quantity of bioactive phytochemicals such as alkaloids, flavonoids in these plants might be responsible for the observed pharmacological activities.

The antibacterial effects observed from the plant extracts are detailed in Table 6. The antibacterial activity of *Punica granatum* against drug-resistant bacteria has been studied. [17, 18, 19] The methanolic leaf extracts of this plant showed significant activity against *S.aureus,* [20,21] we also observed strong antibacterial activity in this plant extract, along with *Rosa centifolia*.

The leaves of *Punica granatum* abundantly possess tannins (punicalin and punicafolin), flavones (luteolin and apigenin), and glycosides which might be responsible for their antibacterial activity. [22] *Punica granatum* possesses numerous medicinal properties. The research study reported that juice, peel or rind, blossoms, leaves, and bark of the pomegranate plant are among the parts that are well-known for their strong anti-inflammatory, antioxidant, and immunomodulatory qualities. These qualities help explain their encouraging potential as anthelmintic, antibacterial, antifungal, antiviral, and anticancer medicines, [23]as well as their antidiabetic and anti-inflammatory possibilities. [24, 25]

The rose plant (Rosa spp.) possesses a variety of medicinal properties, including anti-inflammatory, antioxidant, antibacterial, and antidepressant effects; and is commonly used in aromatherapy and natural skincare due to its calming and healing benefits. [26] Methanolic extracts of various parts of Rose such as leaves, stem, petals and roots exhibited effective antimicrobial potential. [27] Significant antimicrobial efficacy of methanolic extract of *Rosa centifolia* observed against *S. aureus* and *Streptococcus spp.* in the present study. Rose contains a variety of secondary metabolites, such as terpenoids, phenols, fatty acids, and amino acids, known to possess significant biological activities. [28] *Eugenia jambolana* and *Jasminum grandiflorum* also exhibited good activity against both gram positive isolates and *Pseudomonas aeruginosa* while *Eugenia jambolana* could not inhibit any other gram-negative bacteria included in the study, which is similar to the previous study. [29,30] Furthermore, a study conducted on guava extract reported its significant antimicrobial activity against Gram-positive bacteria compared to bacteria belonging to gram-negative category. [31] In our study, only *Streptococcus spp.* found sensitive to guava extract and interestingly it was inactive against *S. aureus* which was susceptible to most of the plant extracts. The extract of *Ocimum sanctum* showed the strongest antimicrobial activity against *Staphylococcus aureus*, which is similar to the findings in other studies. [32] Extracts of *Mangifera indica* demonstrated notable activity against gram-positive isolates. Methanolic extract of *Datura stramonium* found moderately effective against gram positive isolates only. The finding of our study also indicates a higher efficacy of most of the plant extracts against gram positive isolates as compared to gram negative bacteria, which is comparable with other studies. [33] The structural differences in bacterial cell walls can hinder the effective diffusion of extracts, as Gram-negative cell walls are comparatively multilayered and complex than those of Gram-positive bacteria thus reducing the effectiveness of antimicrobial agents against them. The *Mangifera indica* and *Eugenia jambolana*, along with *Punica granatum* with antimicrobial properties were also found to be anti-diabetic potential, which may help in regulating the blood sugar levels and hence lowering the risk of infections in individuals with diabetes. [34, 35]

Significant inhibition was observed during MIC determination (Table 8) of *Punica granatum* and *Rosa centifolia* against gram-negative and gram-positive bacteria. The antimicrobial phenolic compounds present in the plant extract can be the cause of this efficacy. The growths of Gram-positive and Gram-negative microorganisms were equally affected by both extracts, which is inconsistent with results obtained by agar diffusion method. This method affects the antibacterial activity of plant compounds. The crude plant extract contains compounds of different polarity; the less polar compounds diffuse slowly through the agar medium and display low inhibition. These compounds may exhibit greater efficacy in the broth dilution method. The agar well diffusion method is also limited in its utility as it only provides qualitative data. [36]

Plant extracts demonstrate the inhibitory effect by various mechanisms. [37] Some researchers suggest that plant extract antimicrobial constituents (terpenoid, alkaloid and phenolic compounds) work through efflux pump inhibition. [38] Others have also described the inhibitory actions of these extracts in terms of hydrogen bond formation with bacterial enzymes, which in turn brought about various changes in membrane permeability and cell wall integrity. [39]

5. LIMITATIONS:

One limitation of this study is that quality-control strains (ATCC) were not included during the antimicrobial assay of plant extracts and antibiotic susceptibility, hence the findings should be considered as preliminary antibacterial screening of crude plants extracts for the clinical isolates. Future studies should include appropriate quality-control strains to improve the reliability and comparability of the results.

6. CONCLUSION

The present study revealed that the Rose (*Rosa centifolia*) and Pomegranate (*Punica granatum*) extracts exhibited the most significant antimicrobial activity against gram-positive and gram-negative bacteria. From these findings, we suggest that plant extracts used in this study may become a source for discovery of novel antibiotics agents from plants, as it can provide an alternative of antibacterial medicines against antibiotic-resistant bacteria.

However, the result of *In vitro* study should not apply directly to the clinical field without an *in vivo* study, thus further *in vivo* research is necessary to evaluate the biological efficacy and toxicity for the potential application of different compound formulations. Determination of a useful formulation can be done after isolation and characterization of the bioactive compounds responsible for the antibiotic activity of these plants. Other factors such as methods of extraction, solvent choice and variations in techniques can also influence the efficacy of plant extracts

List of Abbreviations

ATCC — American Type Culture Collection

CFU — Colony Forming Unit

E. coli — *Escherichia coli*

IZD — Inhibition Zone Diameter

MHA — Mueller–Hinton Agar

MIC — Minimum Inhibitory Concentration

mg/mL — Milligram per Milliliter

mm — Millimeter

µg — Microgram

µL — Microliter

P. aeruginosa — *Pseudomonas aeruginosa*

R — Resistant

S. aureus — *Staphylococcus aureus*

SD — Standard Deviation

UTI — Urinary Tract Infections

WHO — World Health Organization

w/v — Weight per Volume

v/v — Volume per Volume

ZOI — Zone of Inhibition

Authors Details:

[1*]Assistant Professor, Department of Microbiology, Lords Universal College, Goregaon West, Mumbai, 400104, Maharashtra

[2]Discipline of Biochemistry, School of Sciences, Indira Gandhi National Open University, New Delhi-110068

Authors Contribution:

Conceptualization: MJ

Data Collection and literature search: MJ

Writing -Original Draft: MJ

Reviewing & editing: AS

Approval of final manuscript: All authors

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