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Antibiotic Resistance In Diabetic Foot Ulcers And The Antibacterial Potential Of Compounds Isolated From Endophytic Fungi

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Abstract

Background: Diabetic foot ulcers (DFUs) are a serious complication affecting millions globally. Bacterial infections often complicate DFUs, and rising antibiotic resistance presents a significant treatment challenge. This study aimed to identify the bacterial roles in DFUs and their resistance profiles, while exploring the potential of endophytic fungi from mangrove plants as an alternative antibacterial source.

Methods: A total of 233 participants with DFUs were included. Ulcer severity was graded using the Wagner system. Bacteria were isolated from swab samples and identified. Antibiotic susceptibility was determined using the Kirby-Bauer disk diffusion method. Endophytic fungi were isolated from mangrove leaves, but their antibacterial activity is not reported here.

Results: The study identified a high prevalence of DFUs, with Grade II ulcers most common. Staphylococcus aureus and Escherichia coli were the predominant bacteria isolated, often in polymicrobial infections. Alarmingly, the isolated bacteria exhibited high resistance to commonly used antibiotics like Penicillin, Tetracycline, and Erythromycin. This study confirms the high prevalence of DFUs and highlights the growing concern of antibiotic resistance among DFU-causing bacteria. The findings emphasize the urgent need for alternative treatment strategies. Although antioxidant potential of endophytic fungi isolated from mangroves warrants further investigation, their efficacy against the identified DFU pathogens remains unexplored in this study.

Conclusion: The increasing prevalence of DFUs and the alarming levels of antibiotic resistance among responsible bacteria pose a significant clinical challenge. Further research is crucial to explore promising alternative treatment options like endophytic fungi, alongside stricter antibiotic stewardship measures to combat this growing problem and ensure improved patient outcomes

Keywords: DFU, Wagner scale, resistance, susceptibility, endophytic fungi

Introduction

Diabetes-related foot ulcers (DFUs) occur frequently and can significantly have an effect on both patients and the healthcare system. Morbidity and mortality associated with DFU are significant. Diabetic patients with foot ulcers have a 2.5 times greater risk of death [1-2]. Each year, 9.1-26.1 people are estimated to develop DUFs, as reported by the International Diabetes Federation. Over the past few years, the prevalence of DFU has increased significantly, reaching 6.4% globally [3]. Amputation, gangrene, osteomyelitis, and even death can occur once DFU occurs due to the increased vulnerability to different pathogens. Compared to patients with uninfected foot ulcers, diabetic foot ulcers are 50% more likely to require amputation [4]. Infected diabetic foot ulcers are the leading cause of diabetes-related hospital admissions. There have been both major and minor amputations related to DFU aggravated by infection (83% and 96%, respectively). Nigerian researchers discovered that 24.9% of all ulcers are diabetic foot ulcers, with most of them already graded as Wagner type 3 [5]. It is essentially a polymicrobial infection that causes diabetic foot ulcers. By identifying and controlling bacterial infections, many complications of DFUs can be minimized. In order to initiate early antibiotic treatment, pathogens must be identified and their susceptibility patterns. During their lifetime, endophytic fungi are able to colonize internal plant tissues without causing any harm to their hosts. For antimicrobial protection, endophytic fungi produce secondary metabolites that are the same as the host. There have been several previous studies on mangrove endophytic fungi with potential antibacterial activity. A. ilicifolius leaves contain bioactive compounds, making it possible to find endophytic fungi with high bioactivity. To identify diabetic foot ulcer-causing bacteria as well as their antimicrobial sensitivity pattern, this study was conducted on fungi growing on A. ilicifolius leaves to determine if they were active against the native bacteria of diabetic foot ulcer.

Materials and Methods

This cross sectional study was performed in diabetes clinics in Chennai, India. All the diabetic patients were selected after a confirmed diagnosis of diabetic foot ulcer of any grade. The patients who visited the clinics for regular follow ups were selected for the study and informed consent was taken before the start of the study.

Classification of ulcer

The Ulcer Classification System for Diabetic Foot Ulcers by Wagner was used in this study to categorize ulcers. As part of the classification, patients were classified into three categories: Grade 0 (Pre-ulcerative), Grade 1 (Superficial) ulcers, Grade 2 (Deep) ulcers, Grade 3 (Deep ulcers with abscesses, osteomyelitis, and joint sepsis), Grade 4 (local gangrene), and Grade 5 (global gangrene).

Collection of culture

Samples were taken using two sterile swabs soaked in sterile glucose broth from the deepest part of the ulcer. We used a circular motion to take samples with the swab. Gram staining was performed on one swab and culture was performed on the other. Socio-demographic and clinical data were collected using semi-structured questionnaires. To inoculate the samples, blood agar, MacConkey agar, and chocolate agar were used. The next day, the plates were observed for growth after being incubated at 37°C overnight.

Anti-biotic susceptibility test

Clinical Laboratory Standard Institute (CLSI) guidelines 2020 were followed to test the antibiotic susceptibility of the isolate bacteria on Mueller Hinton Agar (MH). Each isolate's inoculum was prepared by emulsifying colonies in sterile saline (0.85%) at 0.5 McFarland turbidity. MH plates were spread evenly with a sterile swab for three minutes before antibiotic discs were applied. Antibiotic discs purchased from Hi Media Ltd., Mumbai, India. Disc diffusion method was used according to CLSI Standards. Incubation was carried out at 35°C for 16-18 hours, and the diameter of the zone of inhibition was measured with a Vernier caliper. The diameters of complete inhibition zone measured and bacteria with zone with less than 7mm were considered as susceptible and greater than 7mm were considered as resistant. The organisms reported as either sensitive, intermediate sensitive or resistant to antimicrobial agents tested. S. aureus ATCC 25923, Enterococcus faecalis ATCC29212, K. pneumoniaee ATCC70063 & P. aeruginosa ATCC 27853 used as control strains respectively.

Isolation of fungi from the leaves of Acanthus

Leaf samples were collected from the forest area near Nilgiri hills of Acanthus ilicifolius. Then carefully selected mature and healthy plants for sampling. Randomly collected plants were brought to the laboratory in sterile bags for further investigation. Afterwards, the leaves were washed in sterile distilled water for 2 minutes, rinsed in 70% ethanol for 1 minute and dipped in NaOCl 5.3% for 5 minutes [6]. The surface sterilization procedure was validated by imprinting the surface sterilized plant part onto nutrient media and was maintained as control. Afterwards, the leaf segments were submerged in 95% ethanol for 30 seconds, 45% sodium hypochlorite solution for 15 seconds, and 95% ethanol for 30 seconds before being rinsed three times with sterile distilled water for 10 seconds and allowed to surface dry. Cut leaf segments into 0.5cm squares were placed on potato dextrose agar plates (PDA). In order to prevent bacteria from growing, streptomycin sulphate (100mg/L) was added. Every day, fungal colonies were monitored for growth. A fresh PDA plate was then used to grow fungi from the samples. It was done by using standard manuals to identify fungi by their shapes, colors, patterns of mycelium, conidia, and types of spores [7-8-9]

Extraction and Isolation of fungal compounds

Cultures of fungal isolates were grown on PDA slants at 28°C. A fungus endophyte growing out of the cut ends was isolated and sub cultured. From isolates with different morphologies, pure cultures were prepared. For 7-10 days in a rotary shaker, these isolates were mass cultured in 10 ml potato dextrose broth at 28°C. Using Whatman No.1 filter paper, mycelia were separated. The mycelia were collected carefully and dried under shade and powdered. This were extracted with MeOH using soxhlet apparatus. This process was performed 3 times and the filtrates were pooled. The filtrates were evaporated under reduced pressure to yield 50 g of extract (MEF). The extract was eluted first using pet ether (100%), followed by the mixture of Pet ether and Ethanol at various ratios (1:0-0:1) and then finally by Ethanol (100%). Fractions obtained from the column were collected and combined on monitoring with TLC. Fractions that generated identical colour or Rf were combined and subjected for antibacterial activity. The best fraction was subjected for column chromatography. The second step of column chromatography was eluted using chloroform and methanol (1:1) to yield 4 sub fractions which are again subjected to antibacterial activity. The

potent fraction of the sub fraction was eluted again using pet ether and ethanol (7:3) to yield sub fractions [10].

Antibacterial Studies

Screening for antibacterial activity of the plant extracts carried out by Micro-broth dilution method. MIC determination was done by micro broth dilution method. In brief, accurately weighed isolated fraction and extract were serially diluted to achieve concentration of 10, 20, 50, 100, 250, 500 µg/ml using MH broth medium. These freshly prepared solutions were stored in the refrigerator at -4oC till use. 100µl these solutions were poured in a 96 well microtitre plate. 5µl of Isolated bacterial cultures (Coliforms ATCC 25, 41867, P. aeruginosa ATCC 27853, Streptococcus pneumonia, ATCC 700603 and E. fecalis ATCC 25922) ($1x10^4$ CFU/ml) were poured into each of the wells and incubated for 18 hours at 37°C. Afterwards, the plates were scanned for absorbance using a microtitre plate ELISA reader at 600nm. The percentage inhibition was calculated using the following formula % inhibition= (Ac-At)/Ac x100 MIC_{50} was calculated at the concentration of isolated fractions and extracts inhibiting the bacterial growth by 50%.

Results

In total, two species of Aspergillus and Fusarium have been isolated from Acanthus ilicifolius leaves.

Collection and processing of diabetic foot wound swab

The table 1 summarizes the demographic data and medical history of 233 patients involved in your study. The demographic data is divided into patient age groups and gender. Within each age group (20-40, 40-60, 60-70, and over 70), the results show the average age and the number of patients for both males and females and the male and female of age 40-60 are more in the participant list which contributed for about nearly 40%. There's a nearly equal distribution between genders, with slightly more than half being male (52.3%). The table also details co-morbidities, which are other pre-existing medical conditions, for these patients. These co-morbidities are abbreviated as PVD (peripheral vascular disease), PN (peripheral neuropathy), HTN (hypertension), CKD (chronic kidney disease), and CD (cardiac diseases). Hypertension appears to be the most common co-morbidity across all ages and genders. Peripheral vascular disease and peripheral neuropathy are also relatively frequent, while cardiac diseases are less prevalent.

Table 1: Patient demographic data and clinical features

Patient age group	Gender	Mean Patient age	No of patients n=233, %	PVD	PN	HTN	CKD	CD
20-40	Male	35.86±3.28	28 (12.01)	9	6	14	3	-
	Female	35.44±4.94	23 (9.87)	6	4	11	2	1
40-60	Male	53.49±4.88	44 (18.88)	15	26	25	8	3
	Female	51.29±3.28	47 (20.17)	12	10	29	7	1
60-70	Male	66.19±2.95	31 (13.30)	11	8	15	3	4
	Female	66.07±1.38	30 (12.87)	9	7	16	4	3
>70	Male	77.18±1.05	19 (8.15)	8	5	9	3	2
	Female	73.25±1.84	11 (4.72)	3	3	4	2	1

In the 20-40 age group, males had 28 cases, with Grade I ulcers being the most common (12 cases), followed by Grade II (10 cases). No Grade III or V ulcers were observed, and 6 cases were

Grade IV as shown in table 2. Females had 23 cases, with Grade I (9 cases) and Grade II (7 cases) being most frequent, followed by Grade III (5 cases) and Grade IV (2 cases). No Grade V ulcers were reported. For the 40-60 age group, males had 44 cases, predominantly Grade II (20 cases) and Grade I (16 cases). There were fewer cases of ulcers scoring a Grade III (4 cases), Grade IV (3 cases), and Grade V (1 case). Females had 47 cases, mostly Grade II (23 cases) and Grade I (15 cases), with some Grade III (7 cases) and Grade IV (2 cases) ulcers. No Grade V ulcers were observed.

In the 60-70 age groups, males had 31 cases, mainly Grade II (18 cases) and Grade I (11 cases), with 2 cases each of Grade III. No Grade IV or V ulcers were reported. Females had 30 cases, with Grade II (17 cases) and Grade I (10 cases) being most common, followed by Grade III (1 case) and Grade V (1 case). No Grade IV ulcers were observed. For those over 70, males had 19 cases, mostly Grade II (11 cases) and Grade I (6 cases), with 2 cases of Grade III. No Grade IV or V ulcers were reported. Females had 11 cases, primarily in Grade I and II (5 cases each) and in Grade III and Grade IV (4 cases each), with 1 case each of Grade III and Grade IV. No Grade V ulcers were observed. Overall, Grade II ulcers were the most prevalent; followed by Grade I. Higher grades (III, IV, and V) were less common, indicating that most patients had less severe ulcers

Table 2: Prevalence of diabetic foot ulcer of various grades

Patient age group	Gender	Wagner ulcer grade				
		I	II	III	IV	V
20-40	Male	12	10	0	6	0
	Female	9	7	5	2	0
40-60	Male	16	20	4	3	1
	Female	15	23	7	2	0
60-70	Male	11	18	2	0	0
	Female	10	17	2	0	1
>70	Male	6	11	2	0	0
	Female	4	5	1	1	0

Characterization of Bacterial isolates: In the present study mixed bacterial flora were obtained. Table 3 provides an overview of the bacterial species present in diabetic foot ulcers, classified by Wagner ulcer grades I to V. It highlights the presence of both monomicrobial and polymicrobial infections. Monomicrobial infections, involving a single bacterial species, were noted in 41.84% of cases (59 cases). These were more common in lower-grade ulcers, with 15 cases of Grade I, 28 cases of Grade II, 10 cases of Grade III, and 6 cases of Grade IV. There were no monomicrobial infections in Grade V ulcers. Polymicrobial infections, involving multiple bacterial species, accounted for 58.15% of cases (82 cases). These infections increased with ulcer severity: 6 cases were reported in Grade I, 52 in Grade II, 13 in Grade III, 9 in Grade IV, and 2 cases were reported in Grade V. Regarding specific bacterial species, Staphylococcus aureus was the most common, found in 24.82% of cases (35 cases), mainly in Grade II (18 cases) and Grade III (8 cases). Escherichia coli was present in 23.40% of cases (33 cases), predominantly in Grade II (15 cases) and Grade III (9 cases). Both Klebsiella pneumoniae and Pseudomonas species were found in 14.18% of cases (20 cases each), with significant presence in Grade II and higher grades. Streptococcus species accounted for 11.34% of cases (16 cases), mostly in lower grades. Less common bacteria included Enterococcus species (8.51%, 12 cases), Proteus species (1.41%, 2 cases), Aspergillus niger (1.41%, 2 cases), and Candida albicans (0.70%, 1 case). This distribution shows that polymicrobial infections and higher-grade ulcers tend to have a more diverse bacterial presence.

Table 3: Type of bacterial species in the various grades of diabetic foot ulcers

Microorganisms	Wagner ulcer grade					Total n, %
	I (n=83)	II (n=111)	III (n=23)	IV (n=15)	V (n=2)	
Monomicrobial	15	28	10	6	0	59 (41.84%)
Polymicrobial	6	52	13	9	2	82 (58.15%)
Staphylococcus aureus	4	18	8	4	1	35 (24.82%)
Escherichia coli	3	15	9	5	1	33 (23.40%)
Klebsiella pneumonia	2	9	4	4	1	20 (14.18%)
Pseudomonas sps	4	7	5	3	1	20 (14.18%)
Streptococcus sps	3	6	4	3	0	16 (11.34%)
Enterococcus sps	2	4	3	2	1	12 (8.51%)
Proteus sps	0	1	1	0	0	2 (1.41%)
Aspergillus niger	0	0	0	1	1	2 (1.41%)
Candida albicans	0	0	0	0	1	1 (0.70%)
	18	60	34	22	7	141

Table 4 details the antibiotic susceptibility of three Gram-positive bacterial species isolated from diabetic foot ulcers: Enterococcus species, Staphylococcus aureus, and Streptococcus species. The table shows the number and percentage of isolates that are resistant (R) and susceptible (S) to various antibiotics. For Enterococcus species (n=12), the highest resistance was observed to Penicillin and Tetracycline, with 91.66% of isolates resistant and only 8.33% susceptible. Erythromycin also showed high resistance, with 83.33% of isolates resistant. Vancomycin had a resistance rate of 66.66%, while Ciprofloxacin showed 41.66% resistance and 58.33% susceptibility. Co-Trimaxazole had an equal distribution of resistance and susceptibility (50% each).Staphylococcus aureus (n=35) exhibited significant resistance to Penicillin, with 80% of isolates resistant and only 20% susceptible. Erythromycin and Tetracycline also showed high resistance rates of 74.28% and 65.71%, respectively. Vancomycin was the most effective antibiotic, with 82.85% of isolates susceptible. Chloramphenicol and Ciprofloxacin had moderate resistance rates of around 51-54%. Streptococcus species (n=16) showed the highest resistance to Erythromycin and Penicillin, with 75% and 75% resistance, respectively. Vancomycin was the most effective antibiotic, with 62.5% of isolates susceptible. Resistance to other antibiotics like Ampicillin, Chloramphenicol, and Ciprofloxacin ranged from 56.25% to 62.5%. Overall, the data indicates that Vancomycin is generally effective against these Gram-positive bacteria, especially for Staphylococcus aureus and Streptococcus species, while Penicillin and Tetracycline face high resistance rates across all three bacterial species.

Table 4: Gram positive bacterial susceptibility to antibiotics

Antibiotic drug	Enterococcus sps (n=12)		Staphylococcus aureus (n=35)		Streptococcus sps (n=16)	
	R (n, %)	S (n, %)	R (n, %)	S (n, %)	R (n, %)	S (n, %)
Ampicillin	7 (58.33%)	5 (41.66%)	22 (62.85%)	13 (37.14%)	9 (56.25%)	6 (37.5%)
Cefotoxime	9 (75%)	3 (25%)	25 (71.42%)	15 (42.85%)	8 (50%)	7 (43.75%)
Chloramphenicol	4 (33.33%)	8 (66.66%)	18 (51.42%)	17 (48.57%)	10 (62.5%)	6 (37.5%)
Ciprofloxacin	5 (41.66%)	7 (58.33%)	19 (54.28%)	16 (45.71%)	9 (56.25%)	6 (37.5%)
Co-Trimaxazole	6 (50%)	6 (50%)	17 (48.57%)	18 (51.42%)	7 (43.75%)	8 (50%)
Erythromycin	10 (83.33%)	2 (16.66%)	26 (74.28%)	9 (25.71%)	12 (75%)	4 (50%)
Gentamycin	10 (83.33%)	2 (16.66%)	17 (48.57%)	18 (51.42%)	10 (62.5%)	6 (25%)
Oxacillin	6 (50%)	6 (50%)	25 (71.42%)	10 (28.57%)	11 (68.75%)	5 (37.5%)
Penicillin	11 (91.66%)	1 (8.33%)	28 (80%)	7 (20%)	12 (75%)	4 (31.25%)

Tetracycline	11 (91.66%)	1 (8.33%)	23 (65.71%)	12 (34.28%)	10 (62.5%)	6 (31.5%)
Vancomycin	8 (66.66%)	4 (33.33%)	6 (17.14%)	29 (82.85%)	6 (37.5%)	10 (62.5%)

Table 5 presents the antibiotic susceptibility profiles of 4 Gram-negative bacteria (Escherichia coli, Klebsiella pneumoniae, Pseudomonas species, and Proteus species) found in diabetic foot ulcers caused by bacteria. For Escherichia coli (n=33), Amikacin showed a susceptibility rate of 60.60%, with 39.39% resistance. Ampicillin was effective in 66.66% of cases, while 33.33% of isolates were resistant. Cefepime had 54.54% susceptibility and 45.45% resistance, and Cefotoxime was less effective, with 42.42% susceptibility and 57.57% resistance. Chloramphenicol showed a high susceptibility rate of 72.72%. Gentamycin was effective against 63.63% of isolates, while Imipenem showed 60.60% susceptibility. Tetracycline and Trimethoprim had higher resistance rates, with 81.81% and 72.72% respectively. Klebsiella pneumoniae (n=20) displayed significant resistance to several antibiotics. Amikacin had a 65% resistance rate, and Ampicillin showed 60% resistance. Cefepime was particularly ineffective, with 75% resistance. Chloramphenicol and Cefotoxime each had 55% and 60% resistance respectively. Gentamycin had an 80% resistance rate, and Imipenem was resisted by 60% of isolates. Both Oxacillin and Tetracycline showed a 70% resistance rate. Trimethoprim had the highest resistance at 85%. For Pseudomonas species (n=20), resistance to Cefepime was the highest at 90%, with only 10% of isolates being susceptible. Amikacin, Ampicillin, and Oxacillin each had a 60% resistance rate. Cefotoxime and Imipenem showed resistance in 55% and 70% of isolates, respectively. Chloramphenicol had the lowest resistance rate of 15%, indicating high efficacy (85% susceptibility). Gentamycin and Piperacillin were effective against 80% of the isolates, while Tetracycline and Trimethoprim showed moderate resistance at 55% and 75%, respectively. Proteus species (n=2) had limited data due to the small sample size. One isolate was resistant and one susceptible to Amikacin, Ampicillin, Chloramphenicol, Gentamycin, Oxacillin, and Trimethoprim. Both isolates were susceptible to Cefotoxime and Imipenem, but resistant to Cefepime and Tetracycline. Overall, the data indicates variability in antibiotic resistance among different Gram-negative bacteria. Escherichia coli and Klebsiella pneumoniae showed high resistance to several common antibiotics, while Pseudomonas species exhibited the highest resistance to Cefepime. Chloramphenicol and Gentamycin were more effective against Pseudomonas species.

Table 5: Gram negative bacterial susceptibility to antibiotics

Antibiotic drug	Escherichia coli (n=33)		Klebsiella Pneumonia (n=20)		Pseudomonas sps (n=20)		Proteus sps (n=2)	
	R (n, %)	S (n, %)	R (n, %)	S (n, %)	R (n, %)	S (n, %)	R (n, %)	S (n, %)
Amikacin	13 (39.39%)	20 (60.60%)	13 (65%)	7 (35%)	12 (60%)	8 (40%)	1 (50%)	1 (50%)
Ampicillin	11 (33.33%)	22 (66.66%)	12 (60%)	8 (40%)	13 (65%)	7 (35%)	1 (50%)	1 (50%)
Cefepime	15 (45.45%)	18 (54.54%)	15 (75%)	5 (25%)	18 (90%)	2 (10%)	2 (100%)	0 (0%)
Cefotoxim	19 (57.57%)	14 (42.42%)	12 (60%)	8 (40%)	11 (55%)	9 (45%)	0 (0%)	2 (100%)
Chloramphenicol	9 (27.27%)	24 (72.72%)	11 (55%)	9 (45%)	3 (15%)	17 (85%)	1 (50%)	1 (50%)
Gentamycin	12 (36.36%)	21 (63.63%)	16 (80%)	4 (20%)	4 (20%)	16 (80%)	1 (50%)	1 (50%)
Imipenem	13 (39.39%)	20 (60.60%)	12 (60%)	8 (40%)	14 (70%)	6 (30%)	0 (0%)	2 (100%)
Oxacillin	10	23	14 (70%)	6 (30%)	12 (60%)	8 (40%)	1	1

	(30.30%)	(69.69%)					(50%)	(50%)
Piperacillin	19 (57.57%)	14 (42.42%)	13 (65%)	7 (35%)	12 (60%)	8 (40%)	1 (50%)	1 (50%)
Tetracycline	27 (81.81%)	6 (18.18%)	13 (65%)	7 (35%)	11 (55%)	9 (45%)	2 (100%)	0 (0%)
Trimethoprim	24 (72.72%)	9 (27.27%)	17 (85%)	3 (15%)	15 (75%)	5 (25%)	1 (50%)	1 (50%)

Table 6 and figure 1 presents the antibacterial activity of various samples, including the methanol extract (MEF), isolated fractions (F1-F5), sub-fractions of Fraction 3 (SF1-SF4), and sub-fractions of SF2 (SSF1 and SSF2), against Staphylococcus aureus. The table includes the percentage inhibition at different concentrations (µg/ml) and the IC50 values, representing the concentration required to inhibit 50% of bacterial growth. Lower IC50 values indicate higher potency. The MEF exhibited inhibition percentages ranging from 15.44% to 68.3% across different concentrations, with an IC50 value of 120.7 µg/ml. Among the isolated fractions, F3 demonstrated the highest potency with the lowest IC50 value of 64.18 µg/ml, followed by F2 and F4 with IC50 values of 130.1 µg/ml and 134.6 µg/ml, respectively. Among the sub-fractions of Fraction 3, SF2 showed the highest potency with an IC50 value of 61.41 µg/ml, followed by SF1, SF3, and SF4. Sub-fractions of SSF2, SSF1 exhibited the highest potency with an IC50 value of 49.91 µg/ml, while SSF2 showed a moderate potency with an IC50 value of 242.1 µg/ml. Overall, F3, SF2, and SSF1 demonstrated the highest potency against S. aureus, suggesting their potential for further exploration as antibacterial agents.

Table 6: Antibacterial activity of extract and isolated fractions on the S. aureus

Sample	Percentage inhibition at Concentration (µg/ml)					
	10	20	50	100	250	500
MEF	15.44	25.88	35.91	47.92	57.99	68.3
F1	17.32	23.54	32.82	42.98	55.51	66.41
F2	19.38	26.82	35.31	45.8	55.32	66.58
F3	21.27	31.56	42.32	60.51	72.44	81.64
F4	19.36	25.74	33.28	44.42	55.81	67.93
F5	16.44	23.21	30.66	38.54	49.78	59.52
SF1	18.48	25.32	34.2	41.75	50.29	62.31
SF2	22.34	32.35	44.32	61.32	71.38	81.37
SF3	16.6	20.38	27.61	36.39	43.72	54.61
SF4	12.69	19.68	25.66	32.25	40.32	50.49
SSF1	28.25	38.41	48.94	61.49	72.69	82.52
SSF2	18.59	22.52	29.32	36.52	43.71	53.39

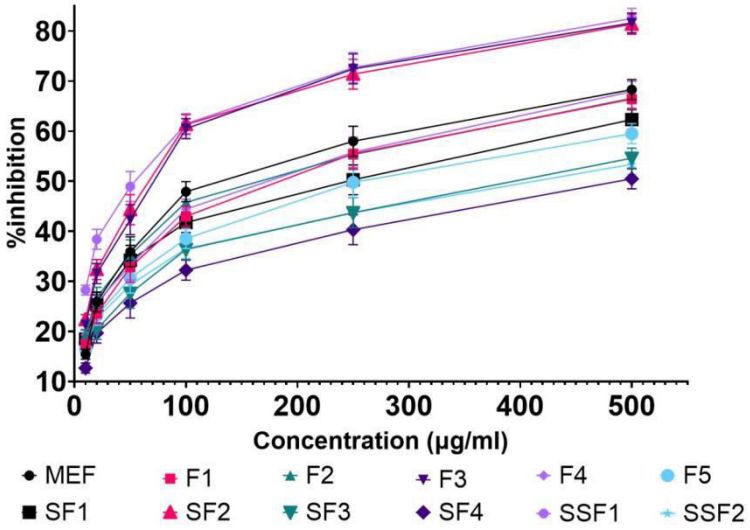

Figure: 1 Antibacterial activity of extract and isolated fractions on the S. aureus

Table 7 and figure 2 outlines the antibacterial activity of the methanol extract (MEF), isolated fractions (F1-F5), sub-fractions of Fraction 3 (SF1-SF4), and sub-fractions of SF2 (SSF1 and SSF2) against Pseudomonas aeruginosa. It includes the percentage inhibition at different concentrations (µg/ml) and the corresponding IC50 values, representing the concentration required to inhibit 50% of bacterial growth. Lower IC50 values signify higher potency. The MEF exhibited inhibition percentages ranging from 15.15% to 62.14% across various concentrations, with an IC50 value of 139.8 µg/ml. Among the isolated fractions, F3 demonstrated the highest potency against P. aeruginosa, with the lowest IC50 value of 73.93 µg/ml. F2 and F4 also displayed moderate potency to the extent that their IC50 values were 165.4 g/ml and 201.01 g/ml, respectively.

Sub-fractions of Fraction 3 (SF1-SF4) showed varying levels of potency. SF2 exhibited the highest potency, exhibiting an IC50 of 63.85µg/ml, indicating strong inhibitory activity against P. aeruginosa. With IC50 values ranging from 218.4 g/ml to 344.01 g/ml, SF1, SF3, and SF4 showed moderate to low potency. Among the sub-fractions of SF2, SSF1 demonstrated the highest potency with an IC50 value of 53.83 µg/ml, suggesting strong inhibitory activity against P. aeruginosa. SSF2 exhibited moderate potency and had an IC50 value of 265.4 g/ml, indicating that it has a moderate effect. Overall, F3, SF2, and SSF1 exhibited the highest potency against P. aeruginosa, as evidenced by their lower IC50 values. These results suggest the potential of these fractions and sub-fractions as antibacterial agents against P. aeruginosa infections.

Table 7: Antibacterial activity of extract and isolated fractions on the P. aeruginosa

Sample	Percentage inhibition at Concentration (µg/ml)					
	10	20	50	100	250	500
MEF	15.15	23.84	35.74	45.87	55.98	62.14
F1	16.12	21.41	28.15	38.95	48.38	59.21
F2	18.12	25.98	32.15	42.78	51.21	60.31
F3	20.14	30.54	41.28	58.41	68.32	75.52
F4	17.14	24.63	32.15	41.23	48.62	52.31
F5	16.32	22.11	29.62	36.25	39.52	42.56
SF1	18.44	25.14	32.14	40.69	45.21	51.21
SF2	21.12	31.25	45.46	60.21	71.24	78.21
SF3	14.52	19.23	28.51	35.21	43.16	54.23
SF4	12.31	18.66	22.46	30.1	38.23	49.26

SSF1	28.14	38.18	47.89	59.21	71.45	80.32
SSF2	18.12	22.22	28.52	35.21	42.21	51.32

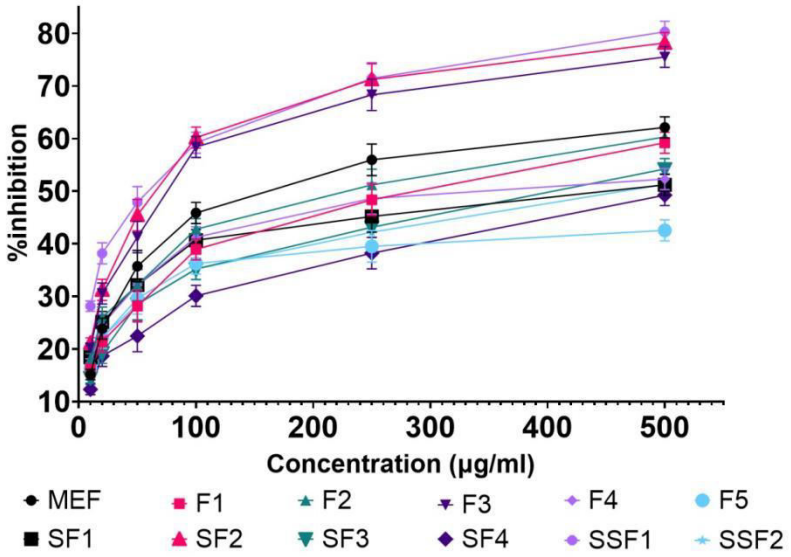

Figure: 2 Antibacterial activities of extract and isolated fractions on the P. aeruginosa

Table 8 illustrates the antibacterial activity of the methanol extract (MEF), isolated fractions (F1-F5), sub-fractions of Fraction 3 (SF1-SF4), and sub-fractions of SF2 (SSF1 and SSF2) against Klebsiella pneumoniae. It includes the percentage inhibition at different concentrations (µg/ml) and their corresponding IC50 values, representing the concentration required to inhibit 50% of bacterial growth. Lower IC50 values indicate higher potency. The MEF showed inhibition percentages ranging from 15.24% to 68.18% across various concentrations, with an IC50 value of 121.4 µg/ml. Among the isolated fractions, F3 exhibited the highest potency against K. pneumoniae, with the lowest IC50 value of 66.95 µg/ml. F2 also displayed notable potency with an IC50 value of 144.3 µg/ml, followed by F1 and F4 which each displayed IC50 values of 145.9 µg/ml and 135.1 µg/ml, respectively (figure 3).

Among the sub-fractions of Fraction 3 (SF1-SF4), SF2 showed the highest potency at 61.0µg/ml, indicating strong inhibitory activity against K. pneumoniae. SF1, SF3, and SF4 exhibited moderate to low potency with IC50 ranges of 161.9 µg/ml to 306.5 µg/ml. In terms of sub-fractions of SF2, SSF1 showed the highest potency with an IC50 value of 50.62 µg/ml, suggesting significant inhibitory activity against K. pneumoniae. SSF2 displayed moderate potency with an IC50 value of 242.3 µg/ml. Overall, F3, SF2, and SSF1 demonstrated the highest potency against K. pneumoniae based on their lower IC50 values. These findings indicate the potential of these fractions and sub-fractions as effective antibacterial agents against K. pneumoniae infections.

Table 8: Antibacterial activity of extract and isolated fractions on the K. pneumoniae

Sample	Percentage inhibition at Concentration (µg/ml)					
	10	20	50	100	250	500
MEF	15.24	25.91	35.78	47.89	57.91	68.18
F1	16.12	23.48	32.75	42.97	55.48	66.32
F2	18.28	26.78	35.25	44.69	53.24	62.41
F3	22.31	31.54	42.28	60.48	69.32	78.62
F4	20.14	25.64	33.18	44.33	55.62	67.84
F5	19.42	23.17	30.62	38.45	45.62	56.41
SF1	21.11	25.24	34.15	41.69	50.21	62.21

SF2	23.61	32.26	44.28	61.24	71.28	81.31
SF3	18.21	20.27	27.54	36.28	43.66	54.38
SF4	13.49	19.64	25.58	32.24	40.23	50.36
SSF1	27.36	38.28	48.89	61.31	72.56	82.42
SSF2	19.52	22.42	29.62	36.31	43.61	53.33

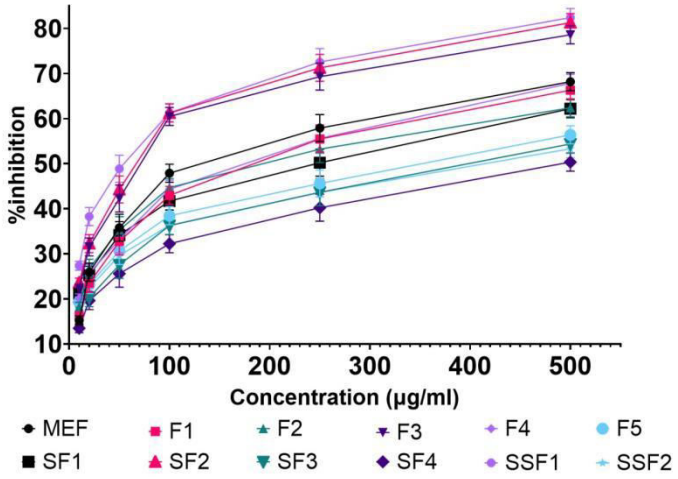

Figure: 3 Antibacterial activity of extract and isolated fractions on the K. pneumoniae

Table 9 and figure 4 showcases the antibacterial activity of the methanol extract (MEF), isolated fractions (F1-F5), sub-fractions of Fraction 3 (SF1-SF4), and sub-fractions of SF2 (SSF1 and SSF2) against Enterococcus faecalis. It presents the percentage inhibition at different concentrations (µg/ml) and their corresponding IC50 values, indicating the concentration required inhibiting 50% of bacterial growth. Lower IC50 values signify higher potency. The MEF demonstrated inhibition percentages ranging from 15.32% to 69.29% across various concentrations, with an IC50 value of 129.8 µg/ml. Among the isolated fractions, F3 displayed the highest potency against E. faecalis, with the lowest IC50 value of 66.60 µg/ml. F2 also exhibited notable potency with an IC50 value of 138.9 µg/ml, followed by F1 and F4 which had IC50 values of 139.3 µg/ml and 129.7 µg/ml, respectively.

Among the sub-fractions of Fraction 3 (SF1-SF4), SF2 showed the strongest inhibitory activity against E. faecalis with an IC50 value of 58.43 µg/ml, indicating strong inhibitory activity against E. faecalis. SF1, SF3, and SF4 exhibited moderate to low potency with IC50 values ranging from 154.8 µg/ml to 283.5 µg/ml. Regarding sub-fractions of SF2, SSF1 showed the highest potency with an IC50 value of 505.52 µg/ml, suggesting significant inhibitory activity against E. faecalis. SSF2 displayed moderate potency with an IC50 value of 241.5µg/ml. Overall, F3, SF2, and SSF1 demonstrated the highest potency against E. faecalis based on their lower IC50 values. These findings suggest the potential of these fractions and sub-fractions as effective antibacterial agents against E. faecalis infections (table 10).

Table 9: Antibacterial activity of extract and isolated fractions on the E. fecalis

Sample	Percentage inhibition at Concentration (µg/ml)					
	10	20	50	100	250	500
MEF	15.32	24.62	33.51	46.87	56.21	69.29
F1	17.28	22.18	33.64	43.98	56.45	67.2
F2	19.31	25.41	36.23	44.21	53.56	65.3
F3	21.24	30.53	43.19	61.41	68.29	79.71
F4	19.32	24.23	34.28	45.41	56.84	68.39
F5	16.41	24.21	31.66	37.55	48.63	60.23

SF1	18.44	24.32	35.23	42.73	51.25	63.32
SF2	22.21	33.23	45.29	62.31	72.30	82.29
SF3	16.58	21.38	28.60	37.29	44.72	55.52
SF4	12.66	20.64	26.32	33.41	41.29	52.39
SSF1	28.18	37.23	49.25	60.49	73.53	83.52
SSF2	18.55	23.41	28.67	35.96	42.85	55.21

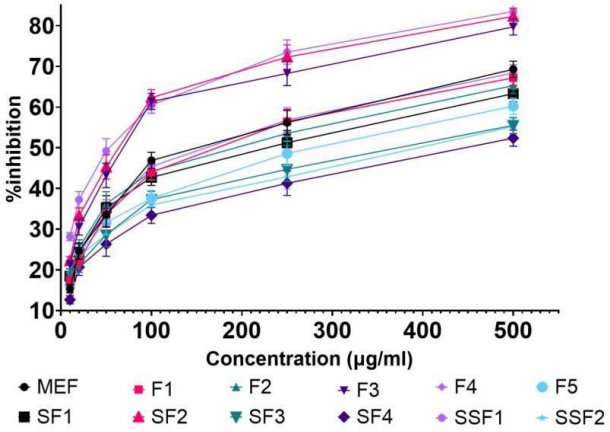

Figure: 4 Antibacterial activity of extract and isolated fractions on the E. fecalis

Table 10: Antibacterial potency of the isolated molecule on the isolated bacteria

Sample	IC_{50}			
	S. aureus	P. aeruginosa	K. pneumoniae	E. fecalis
MEF	120.7	139.8	121.4	129.8
F1	144.8	202.4	145.9	139.3
F2	130.1	165.4	144.3	138.9
F3	64.18	73.93	66.95	66.60
F4	134.6	201.01	135.1	129.7
F5	190.6	320.6	212.5	192.1
SF1	162.8	218.4	161.9	154.8
SF2	61.41	63.85	61.0	58.43
SF3	246.0	254.8	246.8	231.7
SF4	305.5	344.01	306.5	283.5
SSF1	49.91	53.83	50.62	505.52
SSF2	242.1	265.4	242.3	241.5

Discussion

The development of diabetic foot ulcers is a common complication of diabetes. Untreated DFUs pose a major problem if gets infected especially with complications like osteomyelitis, gangrene leading to amputation. DFU were examined for the presence of bacterial infections. The antibiotic susceptibility of the bacteria isolated was evaluated. A partially male dominant sample participated in the study. This may be due to the fact that men are most exposed to the injuries that can lead to ulcers and infections. On top of that, the mean age group of the participants with DFU was found to be 40-60 as supported by other studies that show 51-60 is the median age [11-12].

In our study, diabetic foot ulcers were classified using Wagner's classification. The findings shows that the majority of the ulcers were grade II followed by grade I that is consistent with previous studies [13]. Studies showed that grade III ulcers are prevalent in diabetic patients in abroad especially in African countries. But Indian patients showed significant grade II ulcer which are in line with the current study [14]. The monomicrobial cultures contributed to about 40% of the DFU cultures and majority are the poly microbial cultures meaning which the DFUs are infected with

bacteria of different species. Also over half of the isolated bacteria are from grade II ulcers only. In contrary to other studies [15], study found that the majority of gram positive bacteria are isolated form the DFU as shown in table 3. These findings are in line with previous studies that shows the DFU in India are infected with gram positive bacteria [13-16].

On the other hand, the bacterial cultures isolated from DFU commonly composed of S.aureus, E.Coli, Pseudomonas supported by previous studies showing similar results [15-17]. Especially in Indian population Pseudomonas sps and E.coli are predominant species that are found in the DFU [18]. Though a wide variety of the bacteria were isolated from the ulcers, majority of the species are multidrug resistant against most of the antibiotics tested. S.aureus and Enterococcus are susceptible to chloramphenicol which is in line with the previous study [19].

This high level of resistance is likely due to a variety of factors. As a result of overuse of antibiotics, self-medication, and repeated antibiotic courses associated with chronic DFU, and frequent hospitalizations during follow-up visits, this could occur. DFU infection in the study sites was caused by aerobic pathogenic bacteria ranging in gram-positive as well as gram-negative strains. As confirmed by the current study and their antimicrobial resistance profile may present challenges in the management of patients and result in complications such as limb amputation and osteomyelitis.

In tests, the compound isolated from the fungus showed very good antibacterial potential against 4 bacterial species tested, S. pneumophila ATCC 25923, P. aeruginosa ATCC 27853, K. pneumoniae ATCC 700603 and E. fecalis ATCC 25922, which were common in diabetic foot ulcers. The % inhibitions were calculated and the findings suggests that the isolated fraction 3 from the methanol extract of the fungus showed better activity against all the bacteria and the sub fraction isolated from fraction 3 (SSF1) showed the highest potency against the selected bacteria. As supported by the literature, aspergillus contains coumarins that are potent anti-diabetic drugs [20]. This shows that the SSF1 could be a potential coumarin molecule to establish as an antibacterial compound that can be used to treat diabetic foot ulcers. On the other hand, investigations to be performed to prove the anti-diabetic and wound healing potential of the compound so as to establish the drug as a potential molecule to treat diabetic foot ulcers effectively.

Conclusion

This study highlights the prevalence of diabetic foot ulcers and antibiotic resistance among the bacteria that cause them. While the antibacterial potential of endophytic fungi shows promise, further research is crucial to explore its effectiveness. Developing alternative treatment strategies and implementing stricter antibiotic stewardship programs are critical to combatting this growing challenge and improving patient outcomes. It also briefly touches on the potential solution (endophytic fungi) and emphasizes the need for further research. This leaves the reader with a clear understanding of the problem and the importance of future investigation.

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