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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*

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1. INTRODUCTION

In addition to causing significant health problems to patients, diabetes-related foot ulcers (DFU) have a significant impact on the health care system as well. A significant amount of morbidity and mortality is associated with DFU. The mortality rate for diabetics with foot ulcers is 2.5 times greater [1-2]. Based on the International Diabetes Federation's statistics, 9.1-26.1 people develop DUFs annually. Approximately 6.4% of people worldwide are now infected with DFU, an increase of over 200% since the 1980's. It is possible for DFU to cause amputations, gangrene, osteomyelitis, and even death since

different pathogens are more vulnerable when it occurs. There is a 50% increased risk of diabetic foot ulcers requiring amputation compared to patients with uninfected foot ulcers [4]. Infections associated with diabetic foot ulcers are a leading cause of diabetes-related hospitalizations. Approximately 83 percent of amputations related to DFU aggravated by infection were major, while 96% were minor. In Nigeria, researchers discovered that 24.9% of ulcers are diabetic foot ulcers, with most already being graded Wagner type 3. This infection is primarily a polymicrobial infection. It is possible to minimize the complications of DFUs by identifying and controlling bacterial infections. It is imperative to identify pathogens and their susceptibility patterns in order to initiate antibiotic treatment early. A plant can endure colonization by endophytic fungi for their entire lives without being harmed. In order to protect themselves from bacteria, endophytic fungi produce similar secondary metabolites to those produced by their hosts. Many studies have demonstrated the potential antibacterial activity of mangrove endophytic fungi. A. ilicifolius leaves contain bioactive compounds, making it possible to find endophytic fungi with high bioactivity. In order to identify diabetes-causing bacteria that cause foot ulcers, as well as their antimicrobial sensitivity pattern, this study analyzed fungi growing on A. ilicifolius leaves to determine if they were active against the native bacteria of diabetic foot ulcer.

2. MATERIALS AND METHODS

Several diabetes clinics in Chennai, India participated in this cross-sectional study. Our diabetic patients were all diagnosed with a diabetic foot ulcer of any grade after confirming their diagnosis. Informed consent was obtained from the patients who visited the clinics regularly for follow-ups before the study began.

Classification of ulcer

In this study, ulcers were categorized using Wagner's Ulcer Classification System for Diabetic Foot Ulcers. This classification included three types of ulcers: Grade 0 (Pre-ulcerative), Grade 1 (Superficial), Grade 2 (Deep), Grade 3 (Deep ulcers with abscesses, osteomyelitis, and joint sepsis), Grade 4 (local gangrene), and Grade 5 (global gangrene).

Collection of culture

In sterile glucose broth, we dipped two sterile swabs into the deepest part of the ulcer to collect samples. Swabs were used in a circular motion to collect samples. Two swabs were stained with Gram stain and cultured. Data on socio-demographics and clinical characteristics were collected through semistructured questionnaires. Several types of agar were used to inoculate the samples, including blood agar, MacConkey agar, and chocolate agar. After incubating the plates at 37°C overnight, they were observed the next morning for growth.

Anti-biotic susceptibility test

A test for antibiotic resistance was conducted on Mueller Hinton Agar (MH) using guidelines 2020 published by the Clinical Laboratory Standard Institute (CLSI). Colonies were emulsified in sterile saline (0.85%) at 0.5 McFarland turbidity to form inoculums for each isolate. An antibiotic disc was applied to MH plates after a sterile swab was used to spread them evenly for three minutes. Mumbai-based Hi Media Ltd. provided antibiotic discs. CLSI standards were followed during disc diffusion. An Vernier caliper was used to measure the diameter of the zone of inhibition after incubation at 35°C for 16-18 hours. A bacteria with a diameter of less than 7mm was considered susceptible and one with a diameter of greater than 7mm was considered resistant based on the diameter of the complete inhibition zone. According to the results of the antimicrobial agent test, the organisms were classified as sensitive, intermediately sensitive, and resistant. 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

A leaf sample of Acanthus ilicifolius from the forest around the Nilgiri hills was collected. After selecting mature, healthy plants, samples were taken. To further investigate these plants, random samples were brought to the laboratory in sterile bags. In the following steps, the leaves were first rinsed in 70% ethanol for one minute, then soaked in NaOCl 5.3% for five minutes. In order to determine the effectiveness of the surface sterilization procedure, the surface sterilized plant parts were imprinted onto nutrient media and used as controls.

Extraction and Isolation of fungal compounds

Induction of fungal isolates on PDA slants at 28°C was performed. We isolated and subcultured an endophytic fungus from the cutting ends. We prepared pure cultures from isolates of different morphologies. A mass culture of these isolates was carried out in potato dextrose broth for 7-10 days in a rotary shaker at 28oC for 7-10 days. A Whatman No.1 filter paper was used to separate the mycelia.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.

% inhibition= (Ac-At)/Ac x100

MIC_{50} was calculated at the concentration of isolated fractions and extracts inhibiting the bacterial growth by 50%.

3. 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 116 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 (53.49%). 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=116	PVD	PN	HTN	CKD	CD
20-40	Male	35.86±3.28	14	8	5	13	2	-
	Female	35.44±4.94	11	7	3	10	1	1
40-60	Male	53.49±4.88	22	14	25	24	7	2
	Female	51.29±3.28	23	11	9	28	6	1
60-70	Male	66.19±2.95	15	10	7	14	2	3
	Female	66.07±1.38	15	8	6	15	3	2
>70	Male	77.18±1.05	10	7	4	8	2	1
	Female	73.25±1.84	6	2	2	3	1	1

In the 20-40 age group, males had 35 cases, with Grade I ulcers being the most common (11 cases), followed by Grade II (9 cases). No Grade III or V ulcers were observed, and 5 cases were Grade IV as shown in table 2. Females had 19 cases, with Grade I (8 cases) and Grade II (6 cases) being most frequent, followed by Grade III (4 cases) and Grade IV (1 cases). No Grade V ulcers were reported. For the 40-60 age group, males had 40 cases, predominantly Grade II (19 cases) and Grade I (15 cases). There were fewer cases of ulcers scoring a Grade III (3 cases), Grade IV (2 cases), and Grade V (1 case). Females had 43 cases, mostly Grade II (22 cases) and Grade I (14 cases), with some Grade III (6 cases) and Grade IV (1 cases) ulcers. No Grade V ulcers were observed. In the 60-70 age groups, males had 28 cases, mainly Grade II (17 cases) and Grade I (10 cases), with 1 case of Grade III. No Grade IV or V ulcers were reported. Females had 27 cases, with

Grade II (16 cases) and Grade I (9 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 16 cases, mostly Grade II (10 cases) and Grade I (5 cases), with 1 case of Grade III. No Grade IV or V ulcers were reported. Females had 8 cases, primarily in Grade I and II (3 and 4 case respectively) and with 1 case each in Grade IV. No Grade III and 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	11	19	0	5	0
	Female	8	6	4	1	0
40-60	Male	15	19	3	2	1
	Female	14	22	6	1	0
60-70	Male	10	17	1	0	0
	Female	9	16	1	0	1
>70	Male	5	10	1	0	0
	Female	3	4	0	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 19.33% of cases (29 cases). These were more common in lower-grade ulcers, with 7 cases of Grade I, 14 cases of Grade II, 5 cases of Grade III, and 3 cases of Grade IV. The ulcers of Grade V were not infected with monomicrobial organisms. It was estimated that 39.23% of cases (51 cases) were caused by polymicrobial infections, which involved more than one bacterial species. These infections increased with ulcer severity: 3 cases were reported in Grade I, 26 in Grade II, 16 in Grade III, 4 in Grade IV, and 2 cases were reported in Grade V. Regarding specific bacterial species, *Staphylococcus aureus* was the most common, found in 12% of cases (18 cases), mainly in Grade II (9 cases) and Grade III (4 cases). *Escherichia coli* was present in 10% of cases (15 cases), predominantly in Grade II (7 cases) and Grade III (4 cases). *Klebsiella pneumoniae* species were found in 6.66% of cases (10 cases each), with significant presence in Grade II and higher grades. *Pseudomonas* species were found in 6% of cases (9 cases each), with significant presence in Grade II. *Streptococcus* species accounted for 4.66% of cases (7 cases), mostly in lower grades. Less common bacteria included *Enterococcus* species (4%, 6 cases), *Proteus* species (1.33%, 2 cases), *Aspergillus niger* (1.33%, 2 cases), and *Candida albicans* (0.66%, 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	II	III	IV	V	
Monomicrobial	7	14	5	3	0	29 (19.33%)
Polymicrobial	3	26	16	4	2	51 (39.23%)
Staphylococcus aureus	2	9	4	2	1	18 (12%)
Escherichia coli	1	7	4	2	1	15

						(10%)
Klebsiella pneumonia	1	4	2	2	1	10 (6.66%)
Pseudomonas sps	2	3	2	1	1	9 (6%)
Streptococcus sps	1	3	2	1	0	7 (4.66%)
Enterococcus sps	1	2	1	1	1	6 (4%)
Proteus sps	0	1	1	0	0	2 (1.33%)
Aspergillus niger	0	0	0	1	1	2 (1.33%)
Candida albicans	0	0	0	0	1	1 (0.66%)
	18	69	37	17	9	150

In Table 4, three Gram-positive bacterial species isolated from diabetic foot ulcers are compared for their antibiotic susceptibility: 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=10), the highest resistance was observed to Penicillin and Tetracycline, with 90% of isolates resistant and only 10% susceptible. Erythromycin also showed high resistance, with 80% of isolates resistant. Vancomycin had a resistance rate of 60%, while Ciprofloxacin showed 30% resistance and 70% susceptibility. Co-Trimaxazole had an equal distribution of resistance and susceptibility (50% each). *Staphylococcus aureus* (n=17) exhibited significant resistance to Penicillin, with 94.11% of isolates resistant and only 5.88% susceptible. Erythromycin and Tetracycline also showed high resistance rates of 70.58% and 76.47%, respectively. Vancomycin was the most effective antibiotic, with 88.2% of isolates susceptible. Chloramphenicol and Ciprofloxacin had moderate resistance rates of around 51-64%. *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=10)**		**Staphylococcus aureus (n=17)**		**Streptococcus sps (n=16)**	
	R (n, %)	**S (n, %)**	**R (n, %)**	**S (n, %)**	**R (n, %)**	**S (n, %)**
Ampicillin	8 (80%)	2 (20%)	14 (82.35%)	3 (17.64%)	9 (56.25%)	7 (43.75%)
Cefotoxime	6 (60%)	4 (40%)	15 (88.2%)	2 (11.76%)	9 (56.25%)	7 (43.75%)
Chloramphenicol	4 (40%)	6 (60%)	9 (52.94%)	8 (47.05%)	10 (62.5%)	6 (37.5%)

Ciprofloxacin	3 (30%)	7 (70%)	11 (64.70)	6 (35.29%)	10 (62.5%)	6 (37.5%)
Co-Trimaxazole	5 (50%)	5 (50%)	8 (47.05%)	9 (52.94%)	7 (43.75%)	9 (56.25%)
Erythromycin	8 (80%)	2 (20%)	12 (70.58%)	5 (29.41%)	12 (75%)	4 (25%)
Gentamycin	8 (80%)	2 (20%)	6 (35.29%)	11 (64.70)	10 (62.5%)	6 (37.5%)
Oxacillin	2 (20%)	8 (80%)	10 (58.82%)	7 (41.17%)	11 (68.75%)	5 (31.25%)
Penicillin	9 (90%)	1 (10%)	16 (94.11%)	1 (5.88%)	12 (75%)	4 (25%)
Tetracycline	9 (90%)	1 (10%)	13 (76.47%)	4 (23.52%)	10 (62.5%)	6 (37.5%)
Vancomycin	6 (60%)	4 (40%)	2 (11.76%)	15 (88.2%)	6 (37.5%)	10 (62.5%)

In Table 5, we present the antibiotic susceptibility profiles of four Gram-negative bacteria (Escherichia coli, Klebsiella pneumoniae, Pseudomonas species, and Proteus species). found in diabetic foot ulcers caused by bacteria. For *Escherichia coli* (n=30), Amikacin showed a susceptibility rate of 66.67%, with 33.33% resistance. Ampicillin was effective in 63.33% of cases, while 36.67% of isolates were resistant. Cefepime had 80% susceptibility and 20% resistance, and Cefotoxime was less effective, with 36.67% susceptibility and 63.33% resistance. Chloramphenicol showed a high susceptibility rate of 70%. Gentamycin was effective against 60% of isolates, while Imipenem showed 56.67% susceptibility. Tetracycline and Trimethoprim had higher resistance rates, with 90% and 80% respectively. *Klebsiella pneumoniae* (n=18) displayed significant resistance to several antibiotics. Amikacin had a 72.22% resistance rate, and Ampicillin showed 66.67% resistance. Cefepime was particularly ineffective, with 83.33% resistance. Cefotoxime and Chloramphenicol each had 66.67% and 61.11% resistance respectively. Gentamycin had an 88.89% resistance rate, and Imipenem was resisted by 66.67% of isolates. Oxacillin and Tetracycline showed a 77.78% and 72.22% resistance rate respectively. Trimethoprim had the highest resistance at 94.44%. For Pseudomonas species (n=18), resistance to Cefepime was the highest at 94.44%, with only 5.56% of isolates being susceptible. Amikacin, Ampicillin, and Oxacillin each had a 66.67% resistance rate. Cefotoxime and Imipenem showed resistance in 61.11% and 77.78% of isolates, respectively. Chloramphenicol had the lowest resistance rate of 16.67%, indicating high efficacy (83.33% susceptibility). Gentamycin and Piperacillin were effective against 77.78% and 33.33% of the isolates, while Tetracycline and Trimethoprim showed moderate resistance at 61.11% and 83.33%, 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=30)**		***Klebsiella Pneumonia* (n=18)**		***Pseudomonas* sps (n=18)**		**Proteus sps (n=2)**	
	R (n, %)	**S (n, %)**	**R (n, %)**	**S (n, %)**	**R (n, %)**	**S (n, %)**	**R (n, %)**	**S (n, %)**
Amikacin	10 (33.33%)	20 (66.67%)	13 (72.22%)	5 (27.78%)	12 (66.67%)	6 (33.33%)	1 (50%)	1 (50%)

Ampicillin	11 (36.67%)	19 (63.33%)	12 (66.67%)	6 (33.33%)	12 (66.67%)	6 (33.33%)	1 (50%)	1 (50%)
Cefepime	12 (40%)	18 (60%)	15 (83.33%)	3 (16.67%)	17 (94.44%)	1 (5.56%)	2 (100%)	0 (0%)
Cefotoxim	19 (63.33%)	11 (36.67%)	12 (66.67%)	6 (33.33%)	11 (61.11%)	7 (38.89%)	0 (0%)	2 (100%)
Chloramphenicol	9 (30%)	21 (70%)	11 (61.11%)	7 (38.89%)	3 (16.67%)	15 (83.33%)	1 (50%)	1 (50%)
Gentamycin	12 (40%)	18 (60%)	16 (88.89%)	2 (11.11%)	4 (22.22%)	14 (77.78%)	1 (50%)	1 (50%)
Imipenem	13 (43.33%)	17 (56.67%)	12 (66.67%)	6 (33.33%)	14 (77.78%)	4 (22.22%)	0 (0%)	2 (100%)
Oxacillin	10 (33.33%)	20 (66.67%)	14 (77.78%)	4 (22.22%)	12 (66.67%)	6 (33.33%)	1 (50%)	1 (50%)
Piperacillin	19 (63.33%)	11 (36.67%)	13 (72.22%)	5 (27.78%)	12 (66.67%)	6 (33.33%)	1 (50%)	1 (50%)
Tetracycline	27 (90%)	3 (10%)	13 (72.22%)	5 (27.78%)	11 (61.11%)	7 (38.89%)	2 (100%)	0 (0%)
Trimethoprim	24 (80%)	6 (20%)	17 (94.44%)	1 (5.56%)	15 (83.33%)	3 (16.67%)	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. MEF showed inhibition percentages ranging from 14.45% to 62.32% over different concentrations with an IC50 value of 120.7 µg/ml. Among the isolated fractions, F3 was the most potent with a low IC50 value of 64.18 g/ml, followed by F2 and F4, which each showed a value of 130.1 g/ml and 134.6 g/ml. 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	14.45	24.68	35.11	46.52	55.89	62.32
F1	16.35	25.56	31.80	40.85	51.45	62.22
F2	18.36	26.80	34.21	44.58	54.32	66.58
F3	21.35	30.56	41.22	59.50	70.44	80.64
F4	18.36	24.72	32.26	42.42	54.80	66.92
F5	16.40	22.21	28.66	37.54	48.78	57.54

SF1	17.42	24.36	33.26	40.75	49.27	61.31
SF2	20.34	30.37	40.32	60.32	69.38	82.37
SF3	17.65	19.38	26.61	35.39	42.72	53.61
SF4	12.72	19.65	25.74	32.61	41.32	50.50
SSF1	27.25	39.40	48.94	61.50	72.70	81.50
SSF2	18.60	22.60	30.32	35.52	43.70	55.40

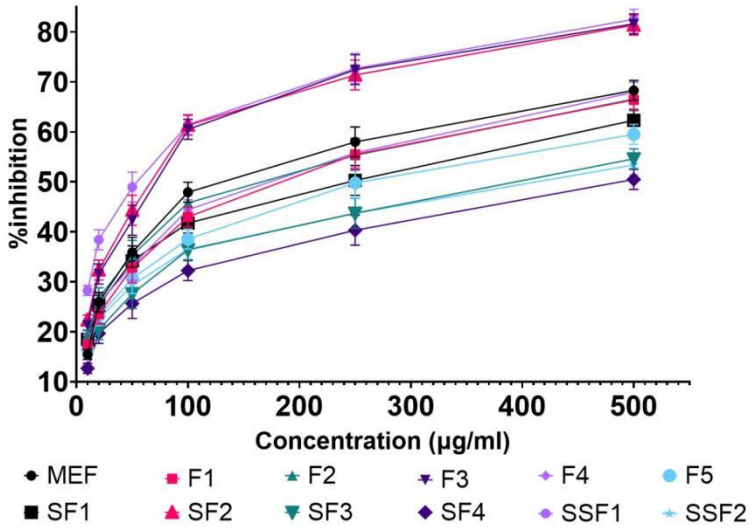

Figure: 1 Antibacterial activity of extract and isolated fractions on the *S. aureus*

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% and 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	15.20	22.80	34.70	42.79	50.95	62.15
F1	16.15	20.41	25.15	35.95	46.38	55.21
F2	17.18	25.01	30.15	44.78	51.21	60.31

F3	20.15	30.53	41.25	56.41	63.32	76.52
F4	17.18	24.66	32.42	41.27	40.62	54.31
F5	16.30	22.08	29.60	36.19	39.58	40.56
SF1	18.45	25.14	32.19	40.70	44.21	48.21
SF2	21.20	30.25	43.46	60.28	70.24	75.21
SF3	14.55	20.23	26.51	36.21	45.16	55.23
SF4	12.36	19.66	22.43	30.17	37.23	45.26
SSF1	28.19	35.18	47.80	59.56	70.45	75.32
SSF2	18.20	22.25	28.50	37.21	42.26	51.30

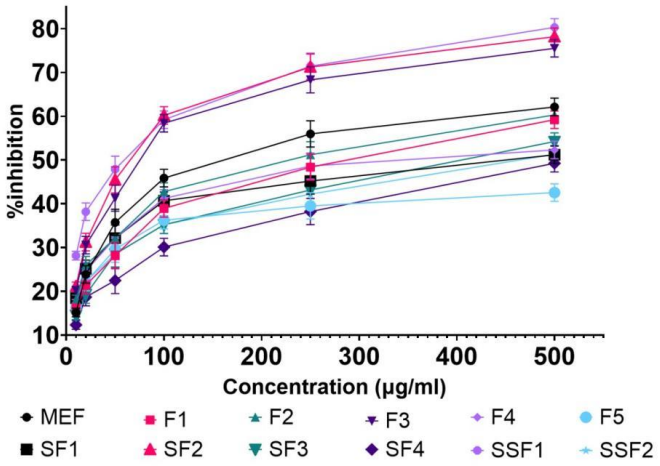

Figure: 2 Antibacterial activities of extract and isolated fractions on the *P. aeruginosa*

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. According to the results, F3 was the most potent fraction when compared with K. pneumoniae and showed the lowest IC50 value at 66.95 µg/ml. It also displayed notable potency, with an IC50 of 144.3 g/ml, followed by F1 and F4 with IC50s of 145.9 g/ml and 135.1 g/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	16.25	25.80	36.71	45.89	57.90	69.18

F1	16.20	24.48	32.72	42.90	55.44	65.32
F2	18.35	26.70	34.25	44.61	53.29	62.35
F3	22.36	30.54	40.28	60.45	69.31	77.62
F4	19.14	25.60	35.18	44.49	55.68	65.84
F5	20.40	23.17	30.60	38.46	45.67	56.45
SF1	21.20	25.20	34.20	41.70	50.23	62.29
SF2	22.60	31.26	44.25	61.25	71.25	81.30
SF3	18.15	20.25	27.52	36.26	43.66	54.41
SF4	13.60	19.68	25.56	32.24	40.48	50.37
SSF1	27.40	38.30	48.85	61.30	72.25	80.42
SSF2	20.52	20.42	29.60	36.28	45.61	55.36

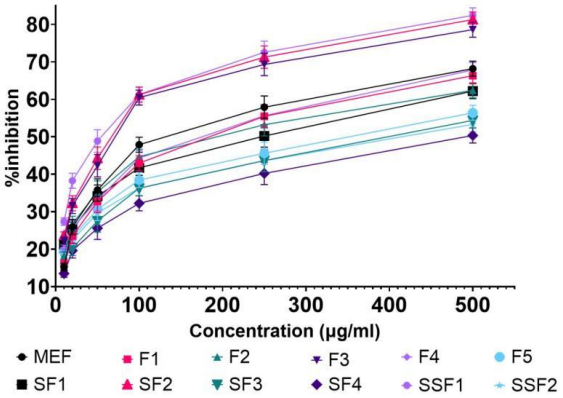

Figure: 3 Antibacterial activity of extract and isolated fractions on the *K. pneumoniae*

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. After the fractions were isolated, F3 showed the lowest IC50 value, 66.60 g/ml. It was followed by F1 and F4 which had IC50 values of 139.3 g/ml and 129.7 g/ml, respectively, with IC50 values of 138.9 and 138.9, respectively, for F2.

According to the results of this study, SF2 was the most potent inhibitor of E. faecalis with an IC50 value of 58.43 milligrams per ml, which indicates strong inhibitory activity on 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	15.30	24.60	35.51	46.82	52.21	69.30

F1	16.25	22.21	33.60	44.08	57.35	67.22
F2	19.35	25.43	35.23	44.69	53.50	64.03
F3	21.26	30.57	43.20	65.41	66.29	80.71
F4	20.32	25.23	34.28	45.14	56.48	68.93
F5	16.48	26.22	31.72	37.51	48.36	62.23
SF1	17.44	24.37	35.28	40.73	51.56	60.32
SF2	22.26	36.23	45.34	62.03	72.03	81.65
SF3	16.64	21.40	28.67	37.92	40.72	55.35
SF4	12.72	20.72	27.32	33.11	41.29	52.40
SSF1	28.48	37.26	51.25	58.49	70.53	82.52
SSF2	18.41	25.41	28.65	35.16	42.85	55.28

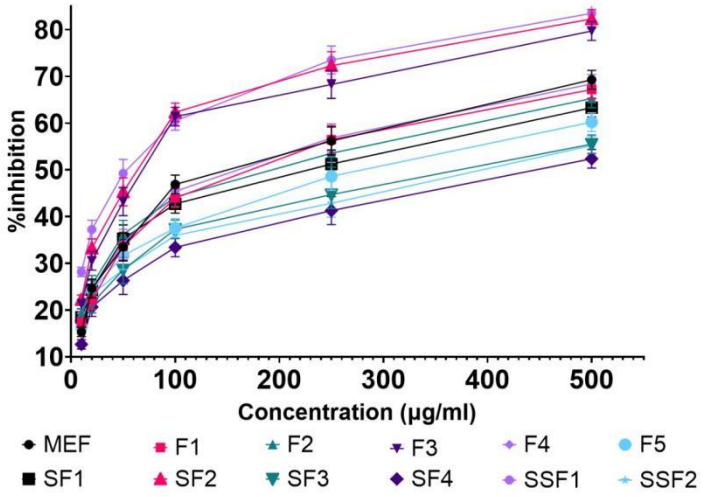

Figure: 4 Antibacterial activity of extract and isolated fractions on the *E. fecalis*

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

Sample	IC_{50}			
	S. aureus	***P. aeruginosa***	***K. pneumoniae***	***E. fecalis***
MEF	118.6	135.8	120.1	130.8
F1	146.8	202.5	143.5	139.6
F2	132.5	165.9	144.2	140.9
F3	65.18	75.93	64.95	66.66
F4	134.8	200.01	132.3	126.8
F5	192.6	321.6	213.5	190.2
SF1	160.8	215.4	160.1	154.6
SF2	65.41	62.85	59.0	58.40

SF3	242.01	246.8	246.2	230.7
SF4	303.2	346.01	306.1	280.5
SSF1	50.90	50.83	51.62	505.01
SSF2	242.0	259.4	240.9	241.50

4. DISCUSSION

In diabetics, foot ulcers are a common complication. It is important to treat DFUs if they get infected, especially if complications arise such as osteomyelitis and 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. During the study, DFU infections were caused by gram-positive and gram-negative aerobic pathogenic bacteria. This study has shown that the antimicrobial resistance profile of these bacteria may present challenges in the management of patients and result in complications such as limb amputations and osteomyelitis.

According to tests, the compound isolated from the fungus showed good antibacterial activity against four bacteria species, 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.

5. CONCLUSION

A study published in this journal highlights diabetic foot ulcer prevalence and antibiotic resistance among the bacteria responsible for 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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