RESEARCH PAPER

Diabetic Foot Ulcers With Antibiotic Resistance And Anti-Bacterial Potential Of Compounds Isolated On Endophytic Fungi

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ABSTRACT
Background: DFU is a high complication that impacts millions of human beings around the world. DFUs are often complicated by bacterial infections and the increasing incidence of antibiotic resistance is a major problem to treatment. This experiment sought to determine the bacterial pathogens present in DFUs in addition to measuring their resistance to antibiotics as well as seeing the viability of endophytic mangrove plant fungi as an alternative source of antibacterial agent. Methods: 233 participants with DFUs were used in the study. The severity of ulcer was measured using Wagner classification system. Swab samples were taken and the bacteria identified the technique. Results: The research found out that the prevalence of DFUs is high with Grade II ulcers being dominant. Isolated bacterial species were mostly polymicrobial, Staphylococcus aureus and Escherichia coli. Worryingly, these were highly resistant bacteria to most of the common antibiotics including Penicillin, Tetracycline and Erythromycin. This observation highlights the increased worry of antibiotic resistance in the infections associated with DFU. Although the antioxidant property of endophytic fungi growing in mangrove deserves further research, the antimicrobial factor of such fungi against any DFU pathogen has not been investigated in this case. Conclusion: More studies are also needed to find alternative treatment methods, like endophytic fungi, and to enforce more rigorous policies of antibiotic stewardship in order to address this growing issue and enhance patient outcomes..

Keywords: Diabetic, Foot Ulcers, Antibiotic Resistance, Endophytic Fungi.
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INTRODUCTION

DFUs are the most widespread and severe effects of diabetes, as they occur in a considerable percentage of patients with this condition on a global scale. A foot ulcer is a skin tear which may spread to other tissues and in case of diabetes, it is usually a combination of factors such as peripheral neuropathy, poor circulation, and immune deficiency [1, 2]. These ulcers are one of the biggest health care system challenges because their pathophysiology is complicated, treatment course is long, and the chances of infections, amputations, and other serious complications are high.

Peripheral neuropathy and peripheral vascular disease are two main causes of the creation of foot ulcers that can be caused by diabetes. The condition is peripheral neuropathy, which is an ailment associated with the persistent elevated level of blood sugar that causes the body of the nerves to degenerate, the legs and lower limbs being the most affected part of the body. This neurological damage causes alterations of sensation, where the people are not able to feel the pain, heat and harm. The slight injuries or blisters may be overlooked and the ulcers occur [3-5]. Also, persons with diabetic neuropathy are vulnerable to foot deformities like Charcot foot where bones become weak, crumbly and deformed, and form new points of pressure where the ulcers are further predisposed to occur. Conversely, peripheral vascular disease, where the blood vessels are affected, influences the circulation of blood to the feet, hampering wound healing, and making the body more difficult in combating infections. Close monitoring of the blood sugar level can go a long way in avoiding the occurrence of ulcers and also when the ulcers have occurred, healing of the ulcers can be enhanced. Besides blood sugar control, it should be properly wounded. This includes the cleaning, debriding, and proper dressing to ensure the wound is kept in a moist environment to guarantee healing. Under certain instances, more complicated treatments like negative pressure wound therapy (NPWT) or growth factor usage can be thought into speeding up the recovery process. The infected ulcers should be treated with antibiotics as soon as possible, and the antibiotic therapy is to be determined by bacterial cultures and susceptibility test outcomes [6]. The empiric antibiotic treatment can be initiated according to the prevalent pathogens related to DFUs, though it is critical to modify the treatment as soon as the results of the culture are revealed to make sure that the infection is properly addressed. Surgical treatment may be required in instances where the underlying bone infection is spread, osteomyelitis, or in instances when the ulcer fails to heal despite conservative treatment.

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MATERIALS AND METHODS

The patients that were included in the study were diabetic patients who had developed diabetic foot ulcers of any grade following the confirmation of their diagnosis. The patients were informed about the importance of informed consent and provided informed consent before the study started when they used to visit the clinics to receive follow-up appointments.

Culture of Sample Collection:

Two sterile swabs were dipped in sterile glucose broth before being inserted deepest section of the ulcer to take samples. Sampling was done by circular motion of the swabs. Gram stain was used to stain two swabs, which were then cultured. The samples were inoculated in different kinds of agar media such as blood agar, MacConkey agar and chocolate agar. The plates were incubated and then managed overnight at 37^{o}C and then checked the next morning whether there was growth or not.

Antibiotic Susceptibility Testing:

There is also an increased resistance of pneumonia causing bacteria to multi-drug resistant (MDR) strains due to the increased use of antibiotics and this is a major challenge to treatment. In the determination of the most effective antibiotics to be used in the treatment of resistant strains-induced infections, antibiotic susceptibility testing (AST) is also necessary. AST is used to identify the susceptibility or resistance pattern of bacterial isolates by testing against a variety of antibiotics to assist clinicians in the choice of the right therapy. This direct intervention will decrease antibiotic misuse and resistances, promote more effective treatment, and improve patient outcomes, which will, in the long term, reduce the negative impact of MDR pneumonia on the health of the population and address the threat posed by this condition.

Fungi Twigs Fungi: Growth of fungi on the Acanthus Leaves

Fungi growth on Acanthus leaves requires one to first obtain Acanthus leaves that are healthy and mature. Wash the leaves using a weak disinfectant to eliminate the contamination on the surface. Then put the leaves into a sterile petri dish or container with a nutrient-sufficient medium such as potato dextrose agar (PDA) to encourage the growth of fungi. Apply fungal spores or mycelium of a fungal culture or nature to the leaves. The set up should be incubated several days in a controlled environment with a high degree of humidity as well as temperature level and this is usually at 25-28^{o}C where the fungi can colonize the leaves. Monitor growth after some time.

Fungal Compounds Extraction and Isolation:

Induction of the fungal isolates was done in potato dextrose agar (PDA) slants at 28^{o} C. The cutting ends were examined using an endophytic fungus that was isolated and pure cultures were prepared using morphologically different isolates. An isolate mass culture was achieved through potato dextrose broth ten days in a rotary shaker at 28^{o} C. The mycelia and a Soxhlet apparatus was used to extract them in methanol. This was extracted three times, and the combined extract filtrates were dried at low pressure to produce 50g of extract. Further eluted with petroleum R-O-R^{1} (100%), then with a combination of petroleum ether and ethanol in different proportions (1:0 to 0:1) and lastly with ethanol (100%). Thin-layer chromatography (TLC) was used to monitor fractions off the column. A fraction with the same color or Rf was united and subjected to antibacterial activity. This fraction was column chromatographed again with the most powerful fraction. Column chromatography was carried out a second time, this time with a mixture of $CHCl_3$ and C_2H_5O (1:1) and four sub-fractions were obtained, re-tested in terms of antibacterial activity. Additional sub-fractions were then received by eluting the most active sub-fraction with petroleum ether and ethanol mixture (7:3).

Antibacterial Studies

The micro-broth dilution technique was used to determine the antibacterial effect of plant extracts. The fractions and extracts were kept at different concentrations by diluting the isolated ones in a series of dilutions of the Mueller-Hinton broth. They were stored at -4o C until the time of use. To test, 100 µl of each solution was put into a 96-well microtitre plate. After that, 5 µl of bacterial culture (including Coliforms, *P. aeruginosa*, Streptococcus pneumoniae, and *E. faecalis*) was added to each well. The plate was left at 37 o C with a duration of 18 hours. Following incubation the absorbance of the respective wells was recorded at 600 nm on a micro titre plate ELISA reader. The percent inhibition was then obtained and the minimum inhibitory concentration known as MIC50 was then determined as the percentage of the concentration at which the bacteria were inhibited by 50% [7-10].

RESULTS

Acanthus ilicifolius has had two *Aspergillus* and *Fusarium* species isolated off the leaf.

Swab of diabetic foot wound collection and processing

The medical history and demographic of the 116 patients who were used in the study is presented. The demographic statistics will be divided into age and sex. These age groups will include 20-40, 40-60, 60-70 and above 70 with the figures depicting the ave. age, as well as the no. of patients under each gender. The most representative ones were the age group of 40-60, as almost 40 percent of the respondents were in this range. The general population balance is close to an equal one with some slight male dominance (53.49%). It also contains details on the co-morbidities or the existing conditions among the patients. These co-morbidities are PVD, PN, HTN, CKD and CD. The prevalence of co-morbidity as was found was hypertension at all ages and genders. Cardiac diseases were less common but non-peripheral vascular disease and neuropathy were common.

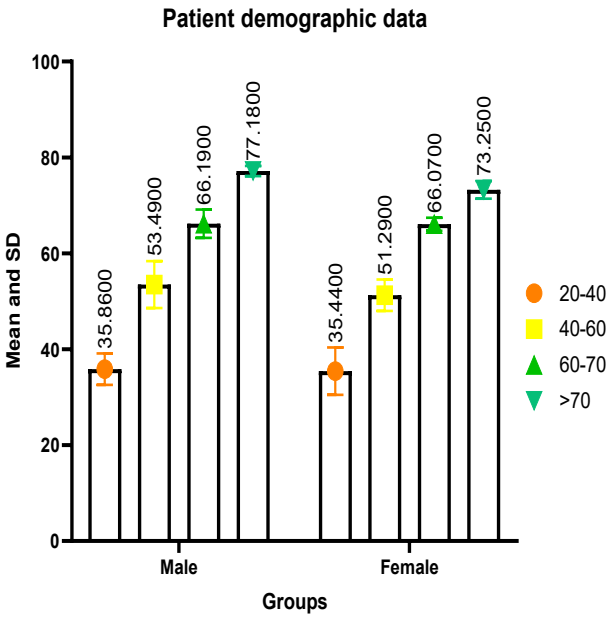

Figure 1: Demographic information and clinical characteristics of patients

The diabetic foot ulcers in the 20-40 age group were found to be more in men with Grade I ulcer being the most prevalent, then Grade II ulcer. No Grade III or V ulcers were found, though Grade IV found in few cases. The same pattern was present in females at this age with Grade I ulcers the most common, Grade II, Grade III, few of Grade IV. None of the Grade V were, however, detected in this group.

The higher incidence of Grade II ulcers in the 40-60 age bracket was followed by Grade I ulcers which were followed by a lower number of higher-grade ulcers. Grade II ulcers were the most prevalent ones among females, and Grade I, as well as high-grade ulcers, were not very numerous.

In males and females, Grade II ulcers were mostly prevalent in the 60-70 age bracket and Grade I ulcers were also prevalent. This age category did not report any Grade IV or V ulcers. Likewise, among the individuals who are above 70 years, Grade II ulcers are most prevalent while Grade I ulcers were also prevalent. Once again, there were low rates of the occurrence of higher-grade ulcers. In general, most of the patients came with less severe ulcers with Grade II being the most common, and Grade I coming next.

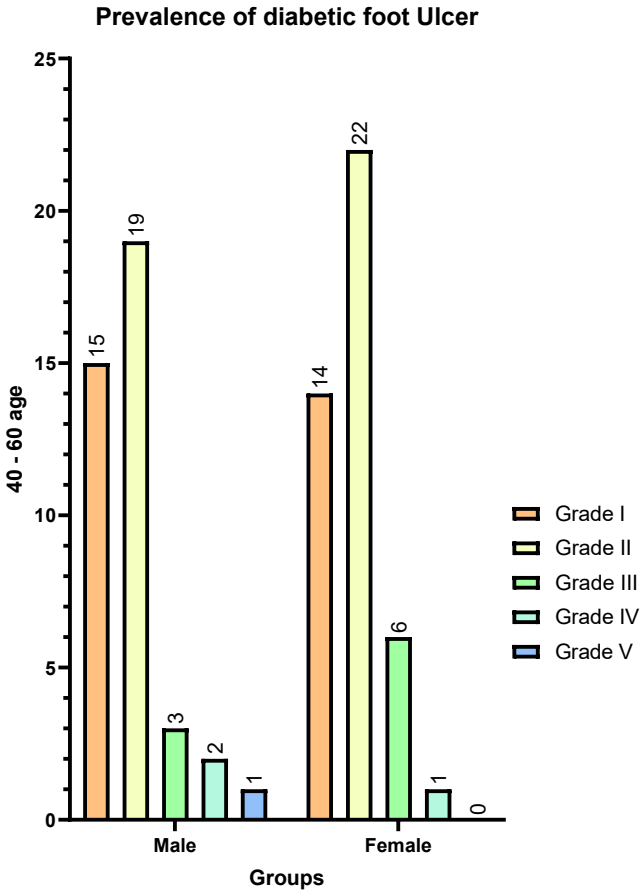

Figure 2: Incidence of diabetic foot ulcer of different grades.

Characterization of Bacterial isolates:

However, diabetic foot ulcers (DFUs) in the current research were observed to be infected by more than forty and forty different bacterial species, both mono and polymicrobial. In lower-grade ulcers, monomicrobial infections, in which only one type of bacteria was present, were more common. The incidence of such infections was also observed to be high in Grade I & II ulcers as compared to Grade III and Grade IV. Interestingly, Grade V ulcers did not have any monomicrobial infections. Conversely, higher-grade ulcers had more polymicrobial infections, which is an infection in multiple bacterial species. These infections were found in a significant proportion of the cases in various grades of ulcers with Grade II having the greatest prevalence.

The most common bacteria that were isolated in DFUs included Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas species. The commonest bacterium was Staphylococcus aureus, which occurred in a considerable percentage of cases, in the Grade II and Grade III ulcers. The Escherichia coli was also widely spread, especially Grade II & III ulcers was highly present in the higher-grade ulcers. Pseudomonas species were also common in Grade II ulcers. Others that were less common were Streptococcus species, Enterococcus species, Proteus species, Aspergillus niger and Candida albicans

with polymicrobial infections more common with the severe grades of ulcers indicating a more complicated bacterial profile.

The susceptibility of three Gram-positive bacteria that are frequent in DFUs to antibiotics (Enterococcus species, Staphylococcus aureus, and Streptococcus species) was also studied. There was also high resistance to Erythromycin with Vancomycin demonstrating superior effectiveness but with some resistance. In the case of Staphylococcus aureus, the resistance rate was high with Penicillin then Tetracycline and Erythromycin. High susceptibility was observed to vancomycin which was found to be the most effective against Staphylococcus aureus. On the same note, Streptococcal species were highly resistant to Erythromycin and Penicillin but Vancomycin was the best antibiotic against this species.

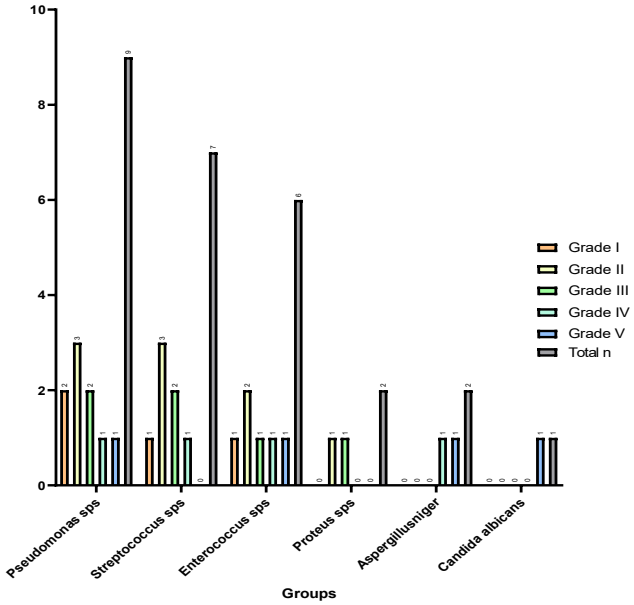

Figure 3: Bacterial species type of diabetic foot ulcers in different grades.

Escherichia coli was also resistant to a number of antibiotics though it was highly sensitive to Amikacin, Ampicillin, and Cefepime. But, the resistance to Tetracycline and Trimethoprim was elevated. Klebsiella pneumoniae was highly resistant to a number of different antibiotics such as Amikacin, Ampicillin, Cefepime, Cefotaxime, and Gentamicin and Imipenem demonstrated much resistance. Pseudomonas species were also very resistant to Cefepime and Amikacin though Chloramphenicol was more effective.

Besides the examination of the bacterial species and the antibiotic resistance profiles, the research conduct also assessed the antibacterial activity of the different plant extracts on Staphylococcus aureus. The Ab efficacy of the C_2H_5OH extract (MEF) and the separated fractions (F1-F5) demonstrated different levels of efficacy. Fraction 3 (F3) was found to be the most effective followed by sub-fraction SF2 which was found to be highly effective in preventing the growth of bacteria. IC50 values of these extracts, which show the level required to suppress the growth of the bacteria by 50 percent, showed that the extracts have the potential to be used as antibacterial agents. In particular, F3 and SF2 showed the most potent evidence indicating they could be utilized further in studying as alternative or adjunctive interventions in DFUs.

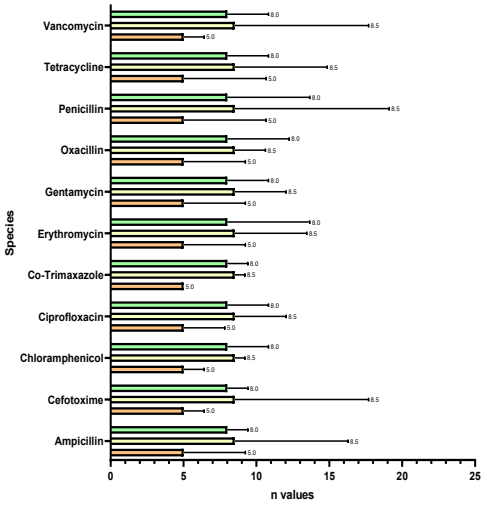

Figure 4: Antibiotic susceptibility of gram positive bacteria

In general, the results indicate the heterogeneity of bacterial infections related to DFUs, and their resistance to the most frequently used antibiotics can be different. This highlights the necessity of other methods of treatment like plant extracts particularly with the increasing cases of antibiotic resistance. The antibacterial effects of these extracts and their use in patients in terms of clinical management should be investigated further, as these extracts may provide some effective adjunctive measures in the prevention and treatment of DFUs.

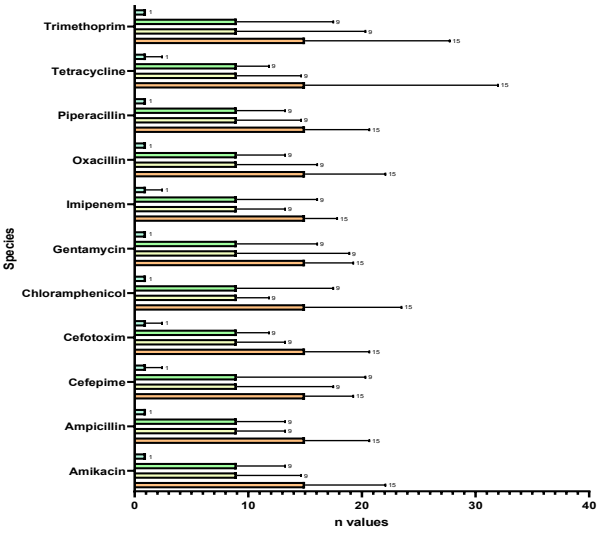

Figure 5: Antibiotic susceptibility of gram negative bacteria.

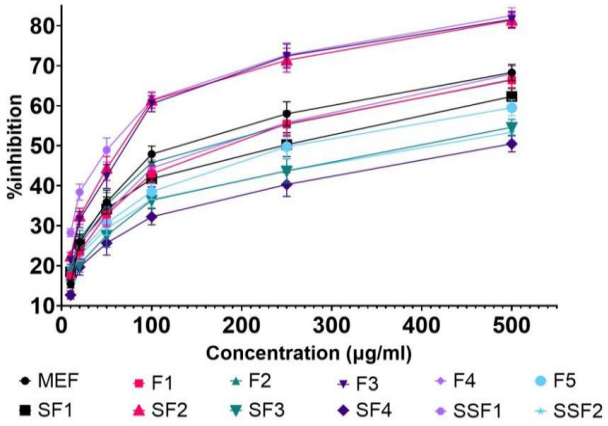

Figure 6: Extract and isolated fractions were preliminary tested to determine antibacterial action on the *S. aureus.*

Figure 7 shows the Ab effects of the C_2H_5OH extract (MEF), the isolated fractions F1-F5, sub-fractions of Fraction 3 (SF1-SF4), and sub-fractions of SF2 (SSF1 and SSF2) on Pseudomonas aeruginosa. The figure shows the % of inhibition at different concentrations (ug/ml) and the IC50 values, which is the concentration needed to inhibit 50% bacterial growth. A decrease in IC50 corresponds to increased potency.

The MEF exhibited a percentage of inhibition between 15.15% and 62.14% at various concentrations with an IC50 of 139.8 3g/ml. F3 showed the greatest potency against *P. aeruginosa* with the lowest IC50 value of 73.93 ug/ml, F2 and F4 had moderate potency with IC50 values of 165.4 ug/ml and 201.01 ug/ml, respectively.

Fractions 3 (SF1-SF4) had different degrees of potency. The potency of SF2 was the most active with the IC50 of 63.85 µg/ml which exhibits a high degree of inhibitory activity against *P. aeruginosa*. Generally, the potency of F3, SF2 and SSF1 was the increased in *P. aeruginosa* with the lowest IC50 values. This implies that these fractions and sub-fractions can have a potential as useful antibacterial agents in the treatment of *P. aeruginosa* infection.

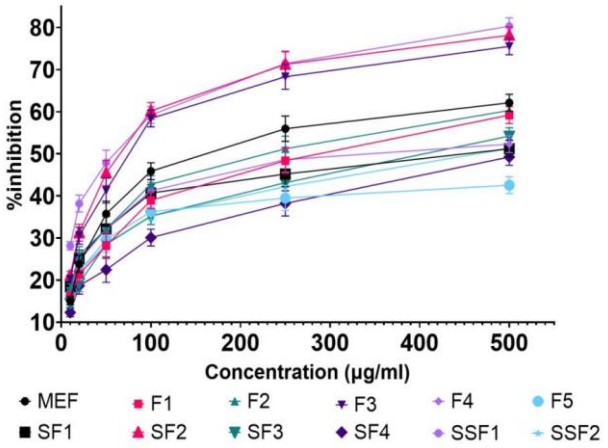

Figure 7: *P. aeruginosa* in terms of antibacterial activity Extracted and isolated

Ab activity (MEF), its separated (F1-F5), and SF3 (SF1-SF4) and Fraction 2 (SSF1 and SSF2) was determined against *Klebsiella pneumoniae*. The MEF showed various inhibitory effects of which its activity varies at different concentrations. Fractions 3 (F3), Fraction 2 (F2), Fraction 1 (F1), and Fraction 4 (F4) represented the strongest antibacterial effect but F3 was the strongest of the isolated fractions. Fractions 3 sub-fractions showed significant inhibitory activities with SF2 being the most powerful. There was also a significant level of activity in SF1 and SSF1 and moderate inhibition in SSF2. Such results indicate that F3, SF2, and SSF1 can be the most promising new therapeutic options to treating the infections of Klebsiella pneumoniae.

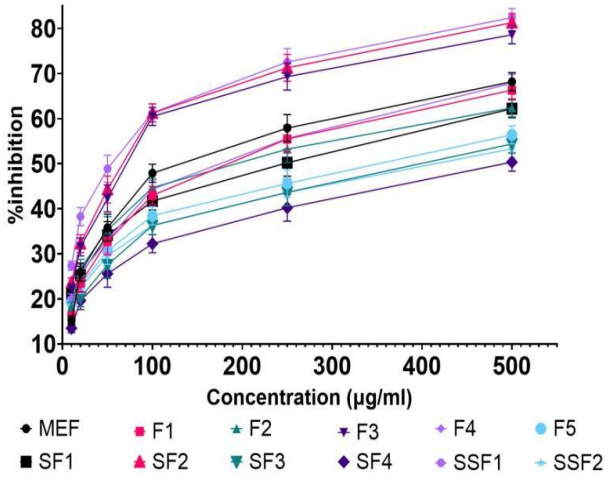

Figure 8: Extracted and isolation fractionally isolated on the *K. pneumoniae.*

The figure gives the percentage of inhibition at different levels and the relative values of IC50; the values of the concentration that was required in order to inhibit half of the bacterial growth. Reduced IC50 means increased antibacterial activity. The MEF was found to have different degrees of inhibition with the greatest activity being observed in the isolated fractions. The strongest antibacterial activity was demonstrated by fraction F3 and then other fractions F1, F4, F2. The sub-fractions produced had SF2 that was the most potent and was highly inhibited. MF1, MF3 and MF4 had moderate and low levels of antibacterial activity. Sub-fraction SSF1 had the best potency and SSF2 had moderate inhibition.

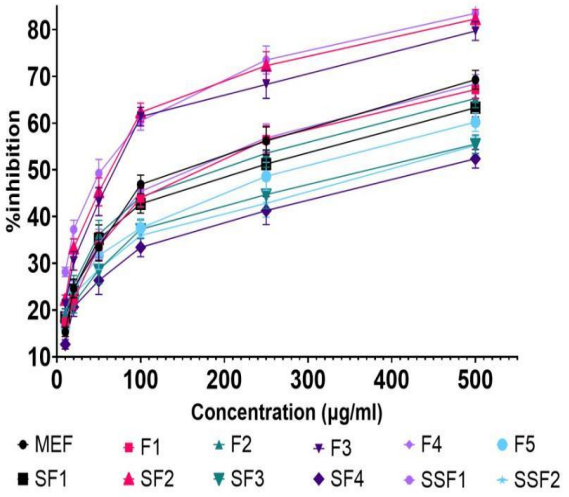

Figure 9: Extract and isolated fractions were tested against the *E. fecalis* in terms of antibacterial activity

DISCUSSION

The frequent complications in diabetic patients. DFUs should be urgently treated in case they get infected particularly when there is gangrene or osteomyelitis, which may result in amputation. This paper analyzed DFUs with bacterial infection and the susceptibility of the bacteria isolated to antibiotics. It was a biased sample which was male dominated, probably because men are more exposed to these types of injury that will cause them to develop ulcers and infections. Also, the average age of the sample of DFU was determined to be between 40-60 years as other studies report the median of 51-60 years [11-12].

In our study, Wagner classification system was used to classify diabetic foot ulcers. The outcome showed that grade II and I ulcers that followed immediately previously comprised the majority [13]. Although grade III ulcers are more common among diabetic patients other than in Africa, namely the African nations, grade II ulcers were found to be very common among the Indian patients in the present study, in line with the existing evidence [14]. The cultures that were obtained of DFUs revealed that approximately 40 percent were monomicrobial, with most being polymicrobial, which means that DFUs were infected by several species of bacteria. It is worth noting that more than fifty percent of the isolated bacteria were grade II ulcers. Unlike the other studies, our study revealed that the bacteria isolated are mostly gram-positive and this explains why previous studies have found that DFUs in mostly infected with gram-positive bacteria.

The bacteria culture taken off DFUs was mainly constituted of S. aureus, E. coli and *P. aeruginosa* that concurs with earlier findings of the same findings [13-17]. Pseudomonas species and E. coli are common in DFUs of the Indian population [18]. Despite the fact that a large number of bacteria were obtained, most of the bacteria were multidrug-resistant to most of the antimicrobial agents used. Both S. aureus and Enterococcus were susceptible to chloramphenicol as it is expected based on past results [19].

The present rate of antibiotic resistance is high, which is probably explained by a number of factors such as excessive use of antibiotics, self-medication, repetitive antibiotics courses that are inherent to chronic DFUs, and frequent hospitalizations on the follow-up visits. The research proved that gram-positive and gram-negative aerobic pathogenic bacteria were the cause of DFU infections. The antimicrobial resistance of these bacteria can be quite challenging in treating the patients and they may cause complications including amputation of limbs and osteomyelitis.

The isolated compounds of a fungal origin presented encouraging antibacterial effect against all the four bacteria species detected in diabetic foot ulcers: *S. pneumoniae*, *P. aeruginosa*, *K. pneumoniae* and *E. faecalis*. Besides, the subfraction obtained in fraction 3 (SSF1) was the most potent against these bacteria. Literature shows that coumarins which are powerful anti-diabetic agents are found in Aspergillus species [20]. This implies that SSF1 might be an effective and useful coumarin molecule as an antibacterial agent which can be applied in managing diabetic foot ulcers. The research studies should be furthered to verify its anti-diabetic and wound-healing effects with the aim of making this compound a possible therapeutic agent that would allow the effective treatment of diabetic foot ulcers [21] [22].

CONCLUSION

The importance of developing alternative treatment measures and applying tougher antibiotic stewardship initiatives would help contain this increasing challenge and enhance patient outcomes. The research also indicates the possibility of using endophytic fungi as a solution and the necessity of further research. This provides an accurate image of the problem to the reader and the importance of the research in the future in order to solve this problem.

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