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Detection of In-Vitro Antibacterial Activity of *Tinospora cordifolia* Leaf Extracts against Multidrug Resistant- *Staphylococcus aureus* Isolated from Diabetic Foot Infections

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ABSTRACT

The manifestations of diabetes are always a great challenge to the medical practitioners and one of its challenging ones are the Diabetic Foot Infection (DFI). There are multiple ways of managing this condition such as using antibiotics to eliminate bacteria and boosting the patient's intrinsic factors, for example, through blood glucose optimization. DFIs are difficult to treat nowadays because of antibiotic resistance; as a result, scientists have turned to medicinal plants for finding drugs against Multi-Drug Resistant(MDR) bacterial strains. *Tinospora cordifolia* is a promising plant with untapped wealth of chemical compounds with high therapeutic potential. These biologically active metabolites work together through different mechanisms causing antibacterial action against the MDR strains. **Objective:** To assess the antibacterial potential of Tinospora cordifolia Leaf Extract (*Tc*LE) against MDR-*Staphylococcus aureus* isolated from pus samples of patients with DFIs. **Methods:** In-vitro experimental study conducted in Ziauddin University from December 2022 to September 2023. Extraction of *Tc*LE was done using a rotary evaporator. The antibacterial activity of *Tc*LE was evaluated by the Agar well diffusion assay. **Results:** Eight different doses were prepared in 10% DMSO using TC's ethanolic leaf extracts. Growth of MDR-*Staphylococcus aureus* strain was inhibited by *Tc*LE at the tested concentration of 250 mg/ml, which was the MIC (the lowest concentration of *Tc*LE which suppressed the growth of MDR-*Staphylococcus aureus* strains). **Conclusions:** *Tc*LE showed antibacterial activity against MDR-*Staphylococcus aureus*, thus establishing it as a potential lead compound source of anti-staphylococci drugs.

INTRODUCTION

Antibiotic resistance is an evolving health problem aggravated by reckless and indiscriminate use of antibiotics. It has resulted in endless struggle between bacteria and new drugs. The microorganisms are now resistant to many drugs. The adverse effects which are the result of this widespread antibiotic resistance are terrifying [1]. The majority of antibiotic resistance mechanisms include enzymatic breakdown or modification, restriction of antibiotic entry into cells to prevent antibiotic accumulation, changes to metabolic pathways, modification of drug-binding sites such as ribosomes to decrease drug efficacy and increased antibiotic efflux from cells. Moreover, bacteria form surface-bound communities called biofilms that make the antibiotics difficult to get inside the bacterial cells. An increasing number of previously treatable diseases have

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become challenging to treat due to multi drug resistance, leading to major clinical challenges. Antibiotics may have more severe adverse effects. This attracts the attention to search for new natural sources of antibacterial agents [2]. The resistant bacterial strains have made the treatment of complications linked with diabetes very difficult. It is estimated that by 2045, about one person in ten shall have diabetes worldwide. As a result of the increased cases of diabetes mellitus in the world, cases of diabetic foot infections are also on the rise. Any infection in the tissue beneath the malleolus in a diabetic person is referred to as a Diabetes Foot Infection, or DFI [3]. 15% of the world's population is suffering from diabetes mellitus, a chronic metabolic disorder, while 19–34% of those who have some form of diabetes, have diabetic foot ulcers. It is estimated that every year, between 9.1 and 26.1 million people will develop DFIs worldwide, putting a heavy burden on any healthcare system because of the recurrent nature of hospitalization and poor healing [4]. *Tinospora cordifolia*, or *TC*, has its place under ayurvedic medicine traditionally. On classification, *TC* falls under the subclass of the larger Angiosperm genus and the family Menispermaceae. Geographically, they are located in the tropical and subtropical regions of Australia, Asia, and Africa. However, the use of *TC* in modern medicine is common for treating rheumatoid arthritis, nausea, headache discomfort, throat infections, flu, diarrhea, stomach ulcers, asthma etc. Anti-stress, antiulcer, anticancer and antitumor actions are a few examples of ethnomedical qualities of *TC*. Various in-vitro studies on agar well diffusion and broth microdilution assays were conducted where *Tc*LE extracted in various solvents was tested against a collection of multidrug-resistant bacteria isolates, such as *Acinetobacter baumannii*, MRSA and *Pseudomonas aeruginosa* [5].

The purpose of this study was to evaluate the antibacterial activity of *Tinospora cordifolia* leaves against MDR-Staphylococcus aureus strains isolated from DFIs by Agar well diffusion method, in light of their well-known antibacterial properties. MIC of *Tc*LE against MDR-*Staphylococcus aureus* using varied concentrations of *Tc*LE was also determined by this method.

METHODS

An in-vitro pre-clinical experimental investigation was conducted at Ziauddin University. The MDR-*Staphylococcus aureus* experimental samples were gathered from Ziauddin Hospital's Microbiology Laboratory. A rotary evaporator was used to extract *TC* leaves. The minimum inhibitory concentration, or MIC, was determined using the Agar well diffusion method. DFI pus samples demonstrating MDR-*Staphylococcus aureus* were included. Agar plates containing additional species growing on them were excluded. The approval for the study was obtained by the Ethical Review Committee, Ziauddin University (Reference code # 60710SMPHA). Fresh leaves of *TC* were obtained from a market in Karachi. The plants were first washed and then kept in the shade for around 15 days to get dry. Plant authentication was performed by a botanist at the Herbarium department; University of Karachi. The specimen of *TC* leaves was given voucher number 97677 respectively. 500 g of air-dried *TC* leaves were mechanically ground into fine powder. The 50g of leaf powder was soaked in 80% ethanol (500 mL). The mixture was then filtered and rotary evaporator was used to remove the solvent and the suspension was stored in airtight bottles. The extraction process was carried out for 48 hours with intermittent pulsating. To create a stock solution, *Tc*LE was dissolved in 10% Dimethyl Sulfoxide (DMSO) [6, 7]. Gram staining, microbiological examination on MacConkey, blood agar and biochemical tests (Catalase test, coagulase test) were performed to identify the isolates of MDR- *Staphylococcus aureus* strains [8]. The identities of MDR- *Staphylococcus aureus* strains were established using a Kirby-Bauer disc diffusion test. MH agar was used to plate antibiotic discs and incubation was done at 37 degrees Celsius for 24 hours. The isolates were characterized as multidrug resistant based on the development of resistance to at least 1 antibiotic in 3 or more different antibiotic groups. For *Staphylococcus aureus*, the following antimicrobial discs were used: Penicillin-10 units, Cefoxitin-30 µg, ciprofloxacin-5 µg, and erythromycin-15 µg discs [9].

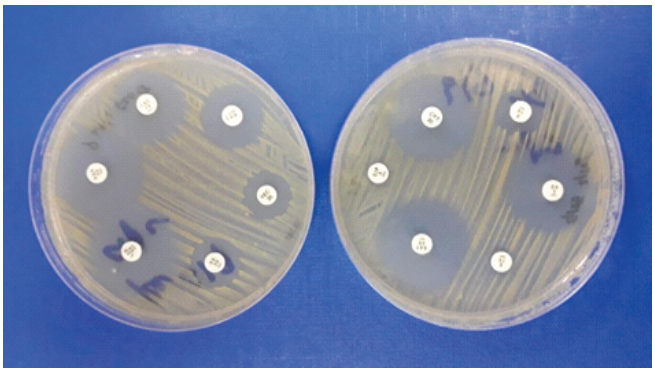

Figure 1: MDR-*Staphylococcus aureus* Identification

After a 24-hour incubation period, it was obtained by selecting the lowest concentration of plant extract that suppressed bacterial growth and was recognized without the need of special equipment [12]. Utilizing the Agar well diffusion method, *TcLE*'s antibacterial effectiveness against MDR-*Staphylococcus aureus* was examined. Generally, colored extracts were preferably assayed using the agar well diffusion method for a number of reasons. Colored extracts will not fully dissolve in the broth medium. This may result in precipitation or aggregation which leads to inconsistent concentrations of active ingredients, which may subsequently affect the reliability and validity of the outcome. The agar medium was particularly useful for studying the diffusion rates of leaf extracts since it was

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stable and uniform; when the latter was placed in an agar, it maintains its structure and supports predictable diffusion patterns [13]. On the surface of MHA plates, the fresh inoculums of MDR bacterial isolates were evenly distributed in comparison to the 0.5 McFarland standards. 50µl of different concentrations of TcLE (3.9 to 500mg/ml) were poured to the wells (6-7mm diameter). The negative control used in the experiment was 10% DMSO. The negative control (DMSO) allowed to distinguish between the solvent's effects and the extract's antimicrobial activity. It was also used as a vehicle control to ensure any observed effects were due to the active ingredient, not the vehicle and to confirm the absence of inherent antimicrobial activity in the medium [14]. Incubation was done at 37°C for 24 to 48 hours after the extract was allowed to diffuse for 15 minutes. The zone of inhibition's presence or absence was assessed after 48 hours. The *TcLE*'s zones of inhibition were assessed and contrasted utilizing a diameter scale [15]. Data analysis was carried out utilizing SPSS version 22.0. Mean and standard deviation were calculated for ZOI. T-test was used in statistical analysis to compare the means of two groups such as experimental(s) versus a control to find if there was a difference in ZOI at varied plant extract concentrations. $P < 0.05$ was considered significant at 95% confidence level.

RESULTS

The growth of MDR-*Staphylococcus aureus* strains was evaluated at 8 concentrations from 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250 and 500 mg/ml made for *Tc*LE, while DMSO was used as negative control. *Tc*LE inhibited the growth of MDR-*Staphylococcus aureus* at two tested concentrations. The MIC was found to be 250mg/ml. The experiment was conducted three times for verification of results.

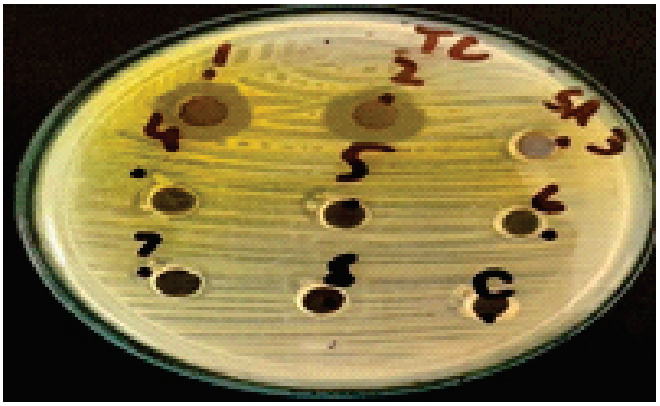

Figure 2: ZOI of *Tinospora cordifolia* Leaf Extract (*Tc*LE) for MDR-*Staphylococcus aureus*. Results of Agar Well Diffusion using MH Agar Plate

Label 1 (500 mg/mL): Zone of Inhibition of 9.6 mm
Label 2 (250 mg/mL): Zone of Inhibition of 7.3 mm.
Label 3-8 (Lower Concentrations): No Inhibitory Effects.
Label C (Control Group): No Inhibitory Effects.

Table 1 depicted that the antibacterial activity of *Tc*LE against MDR-*Staphylococcus aureus* was dose-dependent. The highest concentration used in this study, that was, 500 mg/ml produced a mean zone of inhibition of 9.6 mm, which was genuinely impressive. With 250 mg/ml, the mean ZOI decreased to 7.3 mm. Although it still indicates antibacterial activity, this decrease further implies that the potency of *Tc*LE may be reduced when the concentration was lowered. No inhibitory potential was observed at concentrations lower than 250 mg/ml, the lack of ZOIs, especially for 125 mg/ml and lower concentrations, also establishes this. Thus, the use of higher concentrations has remained an essential aspect of using *Tc*LE for best results as it also signifies that *Tc*LE antibacterial effectiveness was concentration-related.

Table 1: ZOI of *Tinospora cordifolia* Leaf Extract *(TcLE)* for MDR-*Staphylococcus aureus*, Agar Well Diffusion

S. No.	Concentration of Plant Extract used (mg/ml)	ZOI (mm) (Mean ± SD)	p-Value
1.	500	9.6 ± 0.57	*0.045
2.	250 (MIC)	7.3 ± 0.46	
3.	125	-	-
4.	62.5	-	
5.	31.25	-	
6.	15.625	-	
7.	7.8125	-	
8.	3.90	-	
9.	Control	-	

*Comparison of mean ZOIs of *TcLE* against MDR-*Staphylococcus aureus* at various concentrations by T-test

DISCUSSION

The impact of multi-drug resistance microorganisms on worldwide public health has given rise to grave worries. They have inflicted global socioeconomic catastrophe. Antibiotic overuse is making MDR bacteria more common, and nosocomial illnesses brought on by MDR bacteria is on the rise. Wound infections caused by diabetes are the most common, dangerous, and costly infections. It is anticipated that the frequency of DFIs will rise in tandem with the global rise in the incidence of diabetes mellitus. Drug-resistant bacteria, such as MDR-*Staphylococcus aureus* are important contributors of Diabetic Foot Infections (DFIs) [14]. There is a substantial risk of morbidity associated with DFI, leading to lower limb amputation. Diagnosing DFIs can be difficult, which may lead to medication misuse. Because of the polymicrobial nature of DFI and the presence of MDR bacteria, creative antibacterial treatments are needed. Numerous factors, such as geographical features, the intensity of the illness, patient data, and antibiotic use, may influence the bacterial diversity in DFIs [15]. There is an

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infinite and unexplored reservoir of highly medicinal chemical compounds found in plants. A huge variety of these phytochemicals with different structures and functions are utilized to create novel antimicrobials. These phytochemicals have the potential to decrease bacterial resistance processes or act in concert with existing antibiotics to kill bacteria. As an alternative, they might function by obstructing particular molecular targets that are necessary for cell division and proliferation within the cell. Therefore, in order to battle the resistant bacterial diseases, it is essential to develop unique therapeutic medicines using a variety of herbal extracts [16]. Based on the results of Agar well diffusion method, it found that *Tc*LE inhibited the growth of MDR-*Staphylococcus aureus* strains at tested concentrations of 500 mg/ml with a mean ZOI of 9.6 mm and 250 mg/ml with a mean ZOI of 7.3 mm. The MIC of *Tc*LE against MDR- *Staphylococcus aureus* was determined to be 250 mg/ml in this investigation. *TC* is an herbaceous shrub renowned for its medicinal properties. Numerous studies have shown that *Tc*LE has antibacterial qualities against microorganisms, such as MDR-*Staphylococcus aureus*, that can cause potentially fatal infections in humans. Hussein MA *et al.*, evaluated the antibacterial potential of *Tc*LE using the disc diffusion technique. ZOI against Staphylococcus aureus in his study was found to be 25 mm, highlighting the possible antibacterial action of *TC* on sensitive strains and highlighting the need to investigate its activity against MDR strains [17]. The agar-well diffusion method was utilized in an in-vitro investigation to assess the antibacterial activity of *Tc*LE. *Tc*LE's ZOI measured against *Staphylococcus aureus* was 19 mm [18]. Significant amounts of quercetin have been detected in the ethanolic leaf extracts of *TC* [19]. This flavonoid is a remarkable and potent antibacterial agent against drug-resistant strains [20]. There was an isoquinoline alkaloid referred to as berberine which was found in *Tc*LE. It binds to biofilm amyloid proteins, thereby destabilizing its stability, thus *Tc*LE has been in use to combat antibacterial diseases. It was also a potent antibacterial synergist with antibiotics like erythromycin, cefoxitin, and linezolid. Recent studies indicate that berberine can enhance the antibiotics' ability to suppress clinical isolates of MRSA that were resistant to multiple drugs. Based on this kind of evidence, one can deduce from this finding that *Tc*LE might have the ability to be used as a more effective treatment for strains of bacteria that have now become resistant to antibiotics [21, 22]. In an in-vitro study, the phytochemical test of ethanolic extracts of *TC* demonstrated the presence of alkaloids, glycosides, polyphenol, saponins, and terpenoids which have antibacterial properties [23]. These phytoactive compounds present in *Tc*LE can play a very key role in combating MDR bacteria, as they can inhibit the efflux pumps. These compounds interfere with bacterial cell walls, thereby disrupting the integrity of the cell and also inhibiting vital enzymes that play an important role in bacterial life and consequently, their reproduction was inhibited. This makes bacteria prone to environmental stress factors as well as other antibacterial agents [24]. In a study, the anti-biofilm activities of *Tc*LE and the silver nanoparticles from this phytoextract were tested against the biofilm of *S. aureus*. It was observed that both phytoextract from the leaves of *TC* and the biogenic AgNPs from *Tc*LE were successful in reducing the biofilm of S. aureus. Biofilm-producing bacteria produce adhesion molecules that enable them to attach tightly to the extracellular matrix of the wound bed in DFIs, which includes collagen and soft tissues. Bacterial cells, embedded in biofilms, can even become genetically mutated or accumulate resistance genes, contributing to their antimicrobial resistance. This might culminate in the production of subpopulations of bacteria within the biofilm that were highly resistant to various antibiotics. Overall, all these factors contribute to improved tolerance of biofilm-associated bacteria to antibiotic treatment, making the eradication of DFIs more challenging when cases of MDR staphylococci were concerned. It was for such reasons that anti-biofilm activity by *Tc*LE could prove useful in the treatment of DFI by MDR-S. aureus strains [25]. This study also had few limitations. The bioactive compounds of *Tc*LE were not evaluated for the anti-staphylococcal action. The underlying mechanism of action of *Tc*LE against MDR-Staphylococcus aureus strains was not evaluated. Further studies might include experiments to explore whether *Tc*LE could be used in conjunction with other antimicrobial agents to achieve higher efficacy especially at lower concentrations.

CONCLUSIONS

*Tc*LE showed antibacterial potential against MDR resistant *Staphylococcus aureus* and inhibited its growth at 250 mg/ml concentration.

Authors Contribution

Conceptualization: SM[1], AA, MOI
Methodology: SM[1], AA, MOI
Formal analysis: SM[2], HA
Writing, review and editing: SM[1], MK, AA, MOI, SM[2], HA

All authors have read and agreed to the published version of the manuscript

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Conflicts of Interest

All the authors declare no conflict of interest.

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