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Plant Extracts as Potential Antibacterial Agent Against Pathogens Causing Diabetic Foot Ulcers

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Abstract: **Diabetes mellitus is a metabolic disorder that increases blood sugar and the frequent ailment is the complications of lower limbs known as a Diabetic Foot Ulcers (DFU). It is characterized by infection, ulceration, and destruction of the deepest tissues of lower limbs. Infection of DFU with Multi-Drug Resistance microorganism may increase the time of wound healing, hospitalization and patient mortality. The growing menace and spread of antibiotic resistance by different pathogens has led to increased examinations into herbal medicines as alternatives. Medicinal plants as they contain therapeutic value has been used for centuries as remedies for various ailments. The present study deals with the isolation and identification of *Klebsiella pneumoniae,* (MDR) *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli* and *Bacillus subtilis* collected from diabetic wound patients and tested for antibacterial prospects of ethanolic extracts of *Syzygium cumini* and methanolic extracts of *Tinospora cordifolia* and *Gymnema sylvestre.* Antibacterial activity was shown by all the three plant extracts against all the pathogenic agents isolated from diabetic wound samples. There is a slight significant difference found statistically among the three plant extracts against the pathogenic bacteria.**

Index Terms: Antibacterial activity, Diabetic foot ulcers, Diabetes mellitus, Drug resistant bacteria, MIC, Plant extracts and Statistical analysis.

I. INTRODUCTION

Diabetes is a global health issue estimated to rise to over 642 million by 2040 (Maria Luisa Mangoni *et al.,* 2016; William H. Herman, 2017). Bacteria are the most essential factors responsible for causing wound infections in diabetic patients (Ilker Uçkay *et al.,* 2013; Lorina Badger Emeka *et al.,* 2015). In critical conditions it may result into amputation of limbs and hence antibacterial therapy using broad- spectrum antibiotics is repeatedly used. However random use of antibiotics in the long run may lead to the emergence of resistant strains to many antibiotics (Davies & Davies, 2010). Despite the multitude for healing of wound products and technologies that have come out in modern time, the treatment of foot ulcers in diabetic patients still remains a challenge (Guilherme Ferreirade Oliveira *et al.,* 2007). Hence, proper wound management is required to overcome organisms which are resistant to multi drugs which can cause prolonged stay in hospital, causing to higher treatment costs and thereby concomitantly increasing the chances of acquiring other nosocomial infections, paramount to a risk of higher morbidness and impermanence (Wang *et al.,* 2010). Various medicinal plants had been utilized to treat wound infections over the years which offer cost-effective alternative therapy against conventional antibiotics (Mohammed Imran *et al.*,2017).

Medicinal plants are known to be rich source of alkaloids and other phytochemical compounds which can be effectively used as antibacterial agents to treat various ailments. In this research the antibacterial potential (Mariappan senthil kumar, 2015) of *Syzygium cumini* (seeds), *Gymnema sylvestre* (leaves) and *Tinospora cordifolia* (stem) which is (labelled as P1, P2 and P3) have been screened for *K. Pneumoniae* which is resistant to multiple drugs, *S. aureus , P. aeruginosa , P. mirabilis , E. coli and B. subtilis* collected from diabetic patients suffering from wound infections.

Syzygium cumini commonly known as "jambolao", belongs to the family Myrtaceae and one of the most regularly used therapeutic plants to cure diabetes mellitus (Guilherme Ferreira

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de Oliveira *et al.*, 2007). This plant seeds are well known to contain alkaloid, jambolin, jambosine, and glycoside which halts the conversion of starch into sugar (Muniappan Ayyanar*et al.*, 2012). *G. sylvestre* belongs to the family Asclepiadaceae commonly known as "gurmar", used in the ayurvedic system of medicine for its distinct property as sugar destroyer, (Abdul Aziz *et al.*, 2018; Pragya Tiwari *et al.*, 2014). The phytoconstituents like saponins, gymnemic acids and gurmarin are responsible for sweet suppression activity (Pragya Tiwari. *et al.*, 2014). *T. cordifolia* (Menispermaceae) has been identified to contain columbin, tinosporin, and tinosporic acid. It is well familiar to treat various ailments such as inflammations, fevers, skin infections and urinary tract infections etc. (R Jeyachandran *et al.*, 2003; Soham Saha *et al.*, 2012).

II. MATERIALS AND METHODS

A. Collection and Preparation of Plantextracts

Fresh plants parts of leaves of *G. sylvestre* and stem of *T. cordifolia* and seeds of *S. cumini* were collected washed, dried in the air, homogenized to fine powder and labelled as P_1 for *S. cumini*, P_2 for *G. sylvestre* and P_3 for *T. cordifolia* and stored in tightened light protected containers. Preparation of plant extracts was done by adding 20 gm of dried powdered material in 250 ml of solvents packed in Soxhlet apparatus (Shiv Shanker Gautam *et al.*, 2015). Whatman No.1 filter was used to filter the extracts and the solvent was separated from crude extract in vacuum evaporator at 30°C (Sanjay Kumar *et al.*, 2015).

B. Phytochemical analysis

The three crude plant extracts were exposed to identification of the phytochemical compounds detected by standard color tests as adopted earlier (Sathasivam *et al.*, 2008).

C. Test microorganisms and inoculum preparations

The organism identified through Bergey's Manual were *K. pneumoniae* (multi drug resistant bacteria) *S. aureus, P. aeruginosa, P. mirabilis, E. coli* and *B. subtilis* collected from patients suffering from diabetic wound infections from Osmania General hospital, Hyderabad. The isolated strains were maintained at 4°C on nutrient agar slants. Inoculum was made by selecting respective overnight culture grown at 37°C for 24 hrs and the turbidity was adjusted to 0.5Mc Farland standards (Kannan Elangovan *et al.*, 2013).

D. Agar well diffusion method

To evaluate antibacterial activity (Muniappan Ayyanar *et al.*, 2012) of ethanolic extracts of *Syzygium cumini*, methanolic extracts of *Gymnema sylvestre* and *Tinospora cordifolia*, the agar well diffusion method was performed on Mueller Hinton Agar medium (Manish Sharma *et al.*, 2019) against bacterial strain. Broth cultures were spread and wells were made on to the media. 0.1ml of extracts were aseptically transferred to the wells separately and incubated for 24hr at 37°C and the measurement of inhibition zone was recorded. Negative control well was inoculated with DMSO.

E. Minimum inhibitory concentration

MIC is defined as the least concentration of the antimicrobial agent which inhibits the microbial growth after 24 hrs of incubation (Mounyr Balouiri *et al.*, 2016). Bacterial suspension was seeded on to the sterilized Mueller-Hilton agar media and different concentrations (Ashraf *et al.*, 2018) of the effective plant extract (0.01 mg/ml to 1.0 mg/ml) were prepared separately and soaked in sterilized filter paper discs of 5 mm diameter.

The discs soaked with plant extracts were placed on the surface of agar with the aid of sterile forceps and gently pressed down to ensure thorough contact between the disc and agar surface. Sterilized filter paper discs soaked in DMSO was used as negative control and it was incubated at 37°C for 24 hrs. The clear zone of inhibition around the antibiotic disc was measured.

F. Minimum bactericidal concentration

To evaluate bacteriostatic and bactericidal properties of plant extracts against two strains of Gram positive bacteria (*B.subtilis* & *S.aureus*) and three strains of Gram negative bacteria (*E.coli, P.mirabilis* & *P.aeruginosa*) disc diffusion method was performed. The MBC was validated by absence of the tested bacteria growth streaked from inhibition zone corresponding to their lowest MIC's.

G. Data Analysis

The three plant extracts, zone of inhibitions measurement against the pathogens were expressed as mean ± standard error of the mean (SEM). A one-way ANOVA was used to determine significant differences amongst the three plant extracts (P_1, P_2 and P_3). Data were analyzed using the Statistical Package for Social Sciences (SPSS) (version 20.0) software. Followed by comparison test at a significance level of $p<0.05$.by using Tukey's post hoc multiple test.

III. RESULTS AND DISCUSSION

The bacterial culture isolated and identified from the diabetic wound infected patients by morphological and biochemical test were *K.pneumoniae (multidrug resistant bacteria), S. aureus P.aeruginosa, P.mirabilis, E. coli and B.subtilis*. The medicinal plant extracts like *Syzgium cumini* (P_1), *Gymnema sylvestre* (P_2) and *Tinospora cordifolia* (P_3) were used to test against the identified pathogens. Initial screening was done by selecting different solvents for all the three plants extracts since P_1 has shown better results with ethanol, P_2 and P_3 with that of methanol, these two solvents were used to carryout extraction process. A solvent plays an essential role in the proper yield of

plant extraction, the content of chemical components and the tested biological activities (N. Divya *et al.*, 2014; Dieu-Hien Truong *et al.*, 2019). Agar well diffusion was performed to find out the antibacterial activity of plant extracts (N. Divya *et al.*, 2014).

The results were shown that all the three plant extracts were potentially effective in suppressing bacterial growth with variable potency. The phytochemical screening was performed with ethanolic extracts of P_1 and methanolic extract of P_2 and P_3. The presence of bioactive compounds in the plants confers antioxidant and antibacterial activity against the tested pathogens responsible for causing wound infections (Idowu Jonas sagboo *et al.*, 2017). Phytochemical screening of the plants has shown the presence of flavonoids, alkaloids, glycosides, steroids, phenols, tannins and saponins(Kalpna Rakholiya *et al.*, 2014). The phytochemicals analysis of plant extracts is presented in "Table I" Secondary metabolites produced by the plants are known to have beneficial importance in medicinal science (Idowu Jonas Sagbo *et al.*, 2017).

Table I: Phytochemical analysis of plant extracts

S.No	Phytochemical Compounds	P_1	P_2	P_3
1	Terapinoids	-	+	+
2	Saponins	+	+	+
3	Steroids	+	-	-
4	Tannins	+	+	-
5	Carbohydrates	-	+	-
6	Phenols	+	+	-
7	Alkaloids	+	+	-
8	Anthraquinone	-	-	-
9	Flavonoids	+	+	+
10	Glycosides	+	+	-

P1: *Syzygium cumini*, P2: *Gymnema sylvestre*,P3: *Tinospora cordifolia*
+ = Presence, - = Absence

In this research work all the three plant extracts P_1, P_2 and P_3 were subjected to antibacterial activity, as illustrated in "Fig. 1" and "Fig. 2".

Ethanolic extract of P1 has shown good zone of inhibition against *S.aureus* - 27mm, *B.subtilis* - 30mm, *P.mirabilis*- 27mm, *P.aeruginosa*- 21 mm, *E. coli* - 25mm but very less effect on *K.pneumoniae* - 10mm which is multidrug resistant bacteria. Hence the results of P_1 extract revealed that it is better active against Gram positive than compared to Gram negative bacteria.

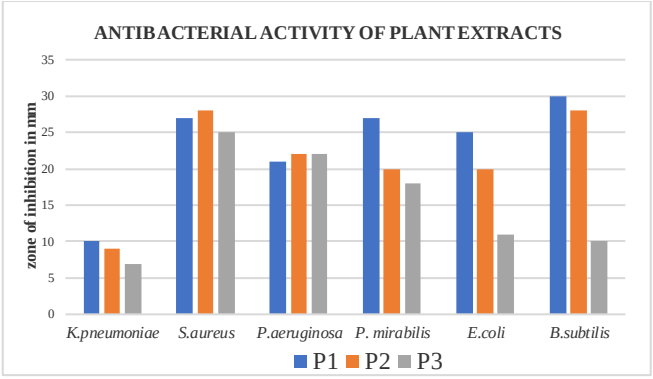

Fig. 1: Comparison of plant extracts P1: *Syzygium cumini*, P2: *Gymnema sylvestre*, P3: *Tinospora cordifolia* against different pathogenic agents determined by agar well diffusion method.

S. aureus remains a prominent etiological agent in pyogenic infections and multi drug resistance, makes the organism an issue for concern and will be devastating if remained untreated (Lorina Badger Emeka *et al.*,2015). According to the literature there is a rise in the resistance to regularly used antibiotics by pathogenic bacteria such as *S. aureus* and *P.aeruginosa* which are significant organisms in causing superficial wounds and leg ulcers (Guilherme Ferreira de Oliveira *et al.*, 2007; Lorina Badger Emeka *et al.*, 2015). Drug resistant strains of *E. coli* are broadly spreaded in hospitals and are progressively being isolated from community acquired infections (Manish Sharma *et al.*, 2009; Flor Y. Ramirez-Castillo *et al.*, 2018). The earlier report has shown *Syzigium cumini* extracts exhibited notable activity against the drug resistant strains of *S. aureus* and *E. coli* (Mohammed Imran *et al.*, 2017). The previous studies done by Niege aracari *et al.*, (2007) shows maximum effectiveness of *Syzygium cumini* leaves hydroalcoholic extract against *Klebsiella*, *Staphylococcus*, *Pseudomonas*, and *Proteus*. Invitro investigation done earlier by Gangadhar A *et al.*, (2011) against strains *E.coli*, *B.subtilis*, *P.aeruginosa* and *S. aureus* (Amol Dagadkhair *et al.*,2007) and inhibitory effect on glucoamylase of seeds of *Syzygium cumini* ethanolic extracts showed moderate to good antibacterial activity. For this reason, researchers are gradually paying their attention to herbal products suchas *Syzigium cumini*, focusing on new better drugs against MDR microbe strains (Braga *et al.*, 2005; Mohammed Imran *et al.*, 2017). The seeds of *Syzygium cumini* stated by Kalpna Rakholiya (2014) possess antioxidant, anti-diabetic, antipyretic, antibacterial, anti- inflammatory, psychopharmacological, hypolipidaemic, and anti- diarrheal activity. (Aziz A, Banerjee S, 2018).

The inhibition zones of methanolic extract of *Gymnema Sylvestre* (P2) as seen in "Fig.1" and "Fig. 2" having maximum inhibitory activity against *S.aureus* - 28mm, *B.subtilis* - 28mm, *P.aeruginosa* - 22 mm, *E. coli* - 20mm, *P.mirabilis* - 20mm but less effective against *K.pneumoniae* - 9mm).

DMSO which was maintained as negative control did not show any inhibition zones and specifying that it is not influencing zone formation. According to previous studies the dried scale leaves of *Gymnema sylvestre* might represents new antimicrobial source with biologically active compound which can be used in modern medicine which helps in healing of wounds (Pragya Tiwari *et al.,* 2014; Soham Saha *et al.,* 2012).

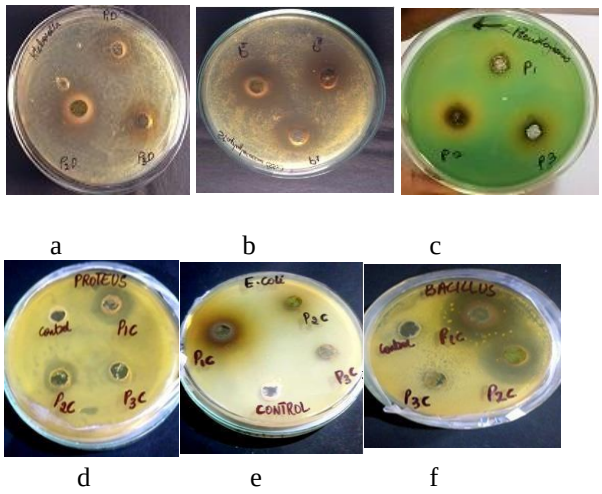

Fig. 2: Antibacterial activity of plant extracts determined by agar well diffusion method. P1: *Syzygium cumini,* P2: *Gymnema sylvestre,* P3: *Tinospora cordifolia*.
a. K.pneumoniae b. S.aureus c. P.aeruginosa
d. P.mirabilis e. E.coli f. B.subtilis

The zone of inhibition (N. Divya *et al.,* 2014) of methanolic extract of *T.cordifolia* (P_3) as seen in "Fig.1 and Fig. 2" shown high inhibitory activity against *S.aureus* - 25mm, *P.aeruginosa* - 22mm, moderate for *P.mirabilis* - 18mm , *E. coli* - 11mm and *B.subtilis* - 10mm but less effective against *K. pneumoniae*- 7mm.

The comparative studies of P_1, P_2 and P_3 plant extracts was done and P_1 was found to be more efficacious than P_2 and P_3 against *K.pneumoniae* which is resistant to multiple drugs. For *S.aureus* and *P.aeruginosa* P_2 was effective followed by P_3 and P_1. *P.mirabilis, E. coli and B.subtilis* culture had shown its maximum sensitivity when tested by P_1 followed by P_2 and P_3. Both Gram positive and Gram negative (Mariappan senthil kumar, 2015) cultures have shown its sensitivity towards all the three plants extracts but the organism which was more sensitive was Gram positive. Results exhibited that multidrug resistant *K.pneumoniae*. showed maximum inhibition by ethanolic extract of P_1 than methanolic extracts of P_2 and P_3. Plant extracts have prominent prospective as antimicrobial compounds against pathogens. Thus, they can be used to treat wounds which are infected by resistant microbes.

The MIC and MBC of the plant extracts (N. Divya *et al.,* 2014) were employed to evaluate bacteriostatic and bactericidal properties by disc diffusion method against wound infected bacteria including Gram positive bacteria (*B.subtilis* & *S.aureus*) and Gram negative bacteria (*E. coli , P.mirabilis* & *P.aeruginosa*). MIC of P_1 extract recorded for *S.aureus, P.aeruginosa and P.mirabilis* is 0.01mg/ml for *E. coli* it has shown 0.02 mg/ml, *B.subtilis* it is 0.03 mg/ml, and *K.pneumoniae* (resistant to multiple drugs) is 3.0mg/ml.

MIC for methanolic extract of P_2 for *P.aeruginosa, B.subtilis* is 0.01 mg/ml, *S.aureus* -0.08 mg/ml and *K.pneumoniae* (resistant to multiple drugs) is 5mg/ml. MIC of methanolic extract of P_3 noted for *S.aureus, P.aeruginosa, E.coli and B.subtilis* is 0.01mg/ml, *P.mirabilis* - 0.05mg/ml and *K.pneumoniae* (resistant to multiple drugs) is 10.0 mg/ml. The MBC was proved by absence of growth of the tested bacteria streaked from inhibitory zone correlating to their lowest MIC's (Ashraf A. *et al.,* 2018). P_1 extract showed potentially bactericidal effect towards the tested pathogenic bacteria (*E.coli* and *P.mirabilis*) while bacteriostatic for *S.aureus, P.aeruginosa, B.subtilis and K. pneumoniae*. While MBC of P_2 extract showed bactericidal effect towards the pathogenic bacteria (*S.aureus, B.subtilis* and *P.mirabilis*) while bacteriostatic for *P.aeruginosa, E.coli* and *K.pneumoniae*. The P_3 extract showed bactericidal effect only towards the *S.aureus,* while bacteriostatic for *E.coli, P.mirabilis, P.aeruginosa, B.subtilisand K.pneumoniae*. The results of MIC and MBC of P_1, P_2 and P_3 plant extracts suggest that these Can be availed to prevent pathogenic bacteria in causing wound infections.

The inhibitory zones of all three plant extracts against the pathogens were indicated as mean ± standard error of the mean (SEM). A one-way ANOVA was applied to determine significant differences amongst the three plant extracts (P_1, P_2 and P_3). Data was evaluated by utilizing the Statistical Package for Social Sciences (SPSS) (version 20.0) software (Mary L McHugh**,** 2011; Sarah Kemp *et al.,* 2011; William lorowitz, 2005**)** followed by Tukey's post hoc multiple comparison test at a significance level of $p<0.05$. The P_1 mean ± SD is recorded as 23.1 ± 6.80 having slightly greater than P_2 value 20.1± 7.46 and for P_3 the values as seen in "Table II" was noted to be 14.9 ±7.06.

There is a slight significant difference found statistically among the three plant extracts against the pathogenic agents.

The plant extract P_1, P_2 and P_3 were effective towards all the pathogens isolated from diabetic wound samples. Since these plant extracts having antiglycemic and antibacterial activity it can be utilized as very potential wound healing agents to treat foot ulcers in diabetic patients.

Table II: Comparison of the Mean Zone of Inhibition of the three plant extracts P1: *Syzygium cumini,* P2: *Gymnema sylvestre,* P3: *Tinospora cordifolia*

Data Summary				
Plant Extracts	N	Mean	Std Dev	Std.Error
P 1	6	23.1111	6.8009	2.7764
P 2	6	20.1667	7.4618	3.0462
P 3	6	14.9444	7.0629	2.8834

As per "Table III" the f-ratio value was recorded to be 2.022 and the P-value is noted as 0.1661.

Table III: P-Value of plant extracts P1: *Syzygium cumini,* P2: *Gymnema sylvestre,* P3: *Tinospora cordifolia.*

Anova Summary					
Source	Degree of freedom	Sum of Squares	Mean Square	F-Stat	P-Value
Between Groups	2	205.273	102.636	2.022	0.1661
Within Groups	15	759.076	50.6051		
Total	17	64.3501	7.0629		

The consequences are not significant at $p < .05$. As shown in "Fig-3". The order of effectiveness is noted to be P_1, P_2 and followed by P_3 extracts.

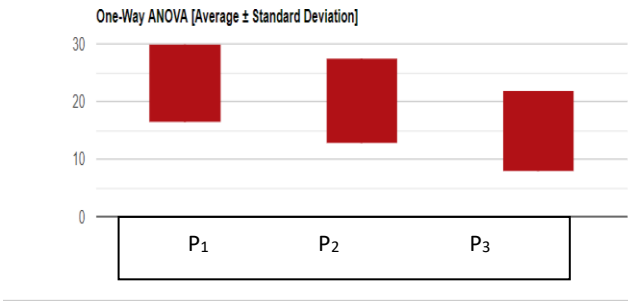

Fig. 3: Average ± standard deviation of plant extracts P1: *Syzygium cumini,* P2: *Gymnema sylvestre,* P3: *Tinospora cordifolia.*

CONCLUSION

The increasing threat and resistance of antibiotics by different types of bacteria has compelled the researchers to look for medicinal plants as an alternative to treat various ailments. Study certifies that *Syzigium cumini*, *Gymnema sylvestre* and *Tinospora cordifolia* are promising source of potential antibacterial agents towards the pathogens accountable for causing wound infections in diabetic patients, including multidrug resistant bacteria (*K.pneumoniae*). Hence these medicinal plants can be utilized as an efficacious wound healing agent in the diabetic patients and also serves as a significant data regarding the valuable research in treating ailments caused by bacteria resistant to multiple drugs.

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