

Antibacterial Activity of *Syzygium aromaticum* (Clove) Against MDR Bacteria Isolated from Diabetic Foot Ulcer and Wound healing

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Abstract: Diabetes mellitus is a chronic lifelong disorder that distinguishes oneself by chronic hyperglycemia and caused damage to organs. Bad control of diabetes leads to skin infections and non-healing foot ulcers which are common in clinical practice. The access to the hospital and mutilation will increase and conversely result in long-term economic, physical, social, and mental disability to the patient. This study is designed to isolate and determine the susceptibility of isolated bacteria to different antibiotics. Drug-resistant bacteria were selected and identified by vitek system 2 and MIC of these isolates were determined. Results revealed that 83 isolates were isolated from 47 patients with diabetic foot infection polymicrobial growth cultures were found in all-patients. Gram negative bacteria showed a high percentage of resistance to tested antibiotics. *Proteus mirabilis* isolate LC587231 record the highest index of antibiotics resistance (0.83). This isolate inhibited by ethanolic extracts of clove at MIC = 12.89 ug/ml and topical application of hydrogel containing clove extract improved wound size, wound index of infected diabetic wound model.

keywords: MDR-*Proteus mirabilis*, *Syzygium aromaticum*, Diabetic foot ulcer, Wound Healing

1.Introduction

Diabetic foot ulcers and infections are common complications associated with diabetic foot disease. These complications are a common cause of morbidity and impose a substantial burden on the patient and society [1]. Infected foot ulcers are the most common cause of diabetes-related hospital admissions and a leading cause of lower amputation [2]. Annually, 9.1 to 26.1 million of population affected with diabetes mellitus (DM) develop a diabetic foot ulcer (DFU) [3].

A diabetic foot infection is defined by the presence of an inflammatory response and tissue injury that can run the clinical spectrum from simple, superficial cellulitis to chronic osteomyelitis as a consequence of the interaction between the host and multiplying bacteria [4].

The host-microbiota interface is often the key point in the development of wound infections. The diabetic foot microbiota come from skin microbiota associated with other clinical statuses [5]. DFUs have been associated with a more polymicrobial microbiota [6,7],

containing more anaerobic bacteria [8] when compared to other wounds.

Ramirez-Acuna et al. [9] reported that DFU has a polymicrobial nature and Jnana et al. [10], showed that the Gram-negative microbes were more abundant in the wound microbiome. Also Mergenhagen et al. [11] isolated 171.822 pathogens from diabetic cultures. MRSA was isolated in 7.5% of cultures and methicillin-susceptible *S. aureus* was isolated in 24.8%. Enterococcus was identified in 14.7% of cultures; *Proteus* in 7.3% and *Pseudomonas* in 6.8% of cultures. Eighty percent of people living in developing countries use traditional medicines which are may only prepared from medicinal plants to meet their primary health care needs [12].

Several medicinal herbal extracts achieved reasonable therapeutic goals regarding the infected DFU [13]. *Syzygium aromaticum* is one of those herbs, belongs to the Myrtaceae family, and exerted broad-spectrum antibacterial activity including MDR-*P. mirabilis* and other beneficial biological activities as, anti-inflammatory antioxidant, etc

[14]. Plant extracts were used in treatment (*in vitro*, local or *in vivo*) of multidrug resistant bacteria isolated from diabetic foot ulcer [15, 16]. Thus, the present work was designed to isolate MDR bacterial and therapeutic potential in a multidrug resistant *Proteus Mirabilis* LC587231 infected diabetes excisional wound model as an antibiotics alternative for DFU therapy.

2. Materials and methods

Sample collection:

Pus samples from the wound of diabetic patients were collected from Zagazig University hospital and also from private clinic called “wound on cell” in Cairo “Nasr city”, Egypt in the period from 6/2017 to 8/2018. Oral informed consent was obtained from the patients before sample collection. Patient’s details were collected using a questionnaire. Pus samples were transported to the laboratory of microbiology in sterile container within one hr. after collection for microbiological analysis.

A total of 47 samples were collected from both inpatients and outpatients. Pus samples were collected by using sterile cotton swabs which are moistened with sterile saline to prevent dryness. For each specimen two swabs were used. One was inoculated on blood agar and another on MacConkey agar plates for isolating the pathogens.

Isolation and purification of bacterial isolates:

Sterile cotton swabs was streaked on MacConkey agar and blood agar plates, after incubation at 37°C for 19-24 hr., the plates were observed for growth and the isolated colonies were identified by morphological, Gram staining and biochemical characteristics. The isolates were identified according to Bergeys Manual of Systematic Bacteriology (1994, 2005) also the antibiotic susceptibility pattern of the isolates was studied by Kirby Bauer’s disc diffusion method [17]. Both broad and narrow spectrum antibiotics were used (13 antibiotics). Isolates that gave the highest % of resistance were selected and identified by vitek 2 system and MIC were determined also by vitek 2 system and the highest MDR index were selected for confirmed identification by molecular 16s

Collection of plants:

Medecinal plants such as *Rosmarinus officinalis*, *Syzygium aromaticum* and *Zingiber officinalis* were collected from various herbalists and markets in Mansoura and Zagazig then confirmed in the Department of Botany, Faculty of Science, Zagazig University.

Preparation of plant extracts:

Collected wild plant materials were washed with sterile water and allowed to drain and dried at 25-30°C in a place not exposed to sunlight and without applying any heat treatment to reduce the loss of active components. Then the dried leaves were crushed to powder and kept in a refrigerator at 4°C until used. Dried, ground leaves (50 gm for each extract) were extracted with 100 ml ethanol by maceration. The extracts were filtered through a Buchner funnel with Whatman filter paper number 3. After filtration, extracts were evaporated under reducing pressure to dryness at 45°C. The collected crude extracts were stored at 4°C until used. All extracts were dissolved in dimethyl sulfide (DMSO 1%). The reconstituted extract solution was sterilized by filtering through 0.45 µm membrane filter before using in bioassay.

Antibacterial activity of extracted plants against MDR isolates:

The pathogens isolated from diabetic foot ulcer sample were inoculated into 10 ml of sterile nutrient broth and incubated at 37°C for 24 h. using a sterile cotton swab, the nutrient broth cultures were aseptically swabbed on sterile Muller Hinton agar plates. Wells of 5 mm in diameter were made aseptically using a good cutter, and 100 µl of ethanol extract of the plant were inoculated. The result was calculated by measuring the inhibition zone in millimeters. For each concentration tested, triplicates were maintained for the confirmation of activity.

Determination of MIC and MBC of plant extracts:

In this experiment, the method of Owuama [18] was used. Briefly sterile test tubes with 1 ml of sterile nutrient broth were prepared. One ml from stock solution prepared from ethanol extract of *Rosmarinus officinalis*, *Syzygium aromaticum* and *Zingiber officinalis* were

transferred to the first tube (1:1), then 1 ml from this tube transferred to 2nd tube (1:2) and this repeated to dilution 1:10 and from last tube 1 ml was decanted. These tubes and control tube (broth only) were inoculated separately by 100 µl of a young culture of *Proteus mirabilis* (1-2 x 10⁸ CFU/ml). After incubation at 37°C for 19-20 hour growth or turbidity were examined using an unaided eye (CLSI, 2012). A tube without growth followed by growth was considered as MIC. From each test tube not showing growth, a loopful of broth was inoculated into nutrient agar plate. These plates were incubated and growth was recorded for the determination of MBC.

***Syzygium aromaticum* and Cefepime Hydrogel preparation.**

The hydrogel was used as a vehicle for topical application of both ethanolic extract and Cefepime antibiotic that formed according to [19], in brief, sodium metabisulfite, methylparaben sodium, and propylparaben sodium were dissolved in water and the Carbopol was added gradually with stirring slowly until a swollen soft gel was developed. The hydrogel was prepared for; Cefepime 20% and clove extract 1.3% (w/w) the concentration was calculated according to the in vitro determined MIC for both Cefepime and clove extract, 0.5 gm of each preparation applied topically on excision type1 diabetic wound model infected with clinical isolates of MDR-*Proteus mirabilis*.

Lab animal:

Forty-five male adult mature *Sprague-Dawley* rats (8:10) week old and weighing (250:300gm) were purchased from Lab. animal house Faculty of Veterinary Medicine Zagazig University. Rats were housed separately in a polypropylene rat cage with standard housing conditions; relative humidity 45: 50 %, 12 hr light/dark cycle, and temperature 22±2 °C. Rats were fed on a standard pelleted diet *ad-libitum* with free access to water throughout the experimental period. Before any experimental procedures lab animals were left one week for acclimatization. All experimental procedures were done following Institutional Animal Care and Use Committee (IACUC), ARRIVE guidelines, and the National Institutes of Health

guide for the care and use of laboratory animals.

Experimental design.

Forty-five male adult mature *Sprague-Dawley* rats were intraperitoneally injected with freshly prepared STZ in 0.1 M citrate buffer at a dose of 65 mg/kg body weight for type 1 diabetes induction according to the method previously described by (King, 2012). Seven days post STZ injection type 1 diabetes onset was assessed via measuring fasting blood glucose using a digital glucometer (U-Right, Korea). Rats that had blood glucose levels above 350 mg /dL were enrolled in the experiment. One week post validation of type1 diabetes onset diabetic wound was done in accordance to Muhammad *et al.* [21], in brief, rats were anesthetized with an intraperitoneal injection of Ketamine 90 mg/kg and xylazine 10 mg/kg. Dorsal fur was clipped with an electrical hair clipper, the skin was disinfected with 70% ethanol, a full-thickness round wound excision 10 mm in diameter and 2 mm in thickness was created with biopsy punch and infected with multidrug-resistant *P. mirabilis* LC587231 isolated from clinical cases of diabetic ulcer at a dose of 2×10⁸ CFU/mL. two days post diabetic wound induction and infection the rats were divided into three groups each of 15 rats; group 1; diabetic infected wound treated with hydrogel only, group 2 infected diabetic wound topically treated with 0.5 gm of Cefepime hydrogel 1%, 2days post infected diabetic wound induction once daily for 2 successive weeks, and group 3; infected diabetic wound topically treated with 0.5 gm ethanolic clove extract hydrogel 5%, 2 days post infected diabetic wound induction once daily for 2 successive weeks (the applied doses were selected according to a dose response pilot study *supplementary data* (Fig. 1). Rats were kept separately on polypropylene cages avoiding fighting and wound biting. To assess the wound healing capacity wound diameter and wound index were evaluated every 3 days throughout 18 days.

Measuring glycemic parameter & oxidant/antioxidant activity.

Blood glucose was measured 7 days post-STZ injection and 18 days post diabetic wound induction with a digital glucometer (U-Right,

Korea). Insulin was measured in serum with sandwich ELISA Kit (SunRedBio, China) according to the method previously described by [22].

Measuring wound diameter and wound index.

Wound diameter was measured with a measuring scale per cm and the wound index was calculated with the following equation [wound diameter of each time set point/initial wound diameter] according to Mendes et al. [22].

Data analysis and statistics.

Statistical analysis was performed by GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, United States). Data expressed as mean $\pm$ standard error mean (SEM). Statistical comparisons were performed using a one-way analysis of variance (ANOVA) test followed by a post hoc Tukey test. The results indicated a statistical significance when $P < 0.05$.

3. Results

From 47 patients with diabetic foot infection in Zagazig University and 83 isolates of

bacteria were collected after growing of samples in both Macckary and blood agar media. Results in Table (1) revealed that all examined patients diabetic foot swaps were gave bacterial growth on both medium (Macc & blood) after incubation at 37°C for 24 hours. The highest number of bacteria isolates 42 (50.60%) were isolated from patients with diabetic foot infection at age range from 56-75 year followed by 36 (43.37%) isolates obtained from patients at age range from 36-55 year and lowest number of isolates 5 (6.02%) were isolated from patients with age range from 16-36. Also female patients with diabetic foot infection recorded the highest number 26 (55.31%), these patients gave bacteria isolates reached to 47 (56.63%). Meanwhile, the male was 21 (44.68%) gave patients 36 bacteria isolates (43.37%).

Gram negative bacteria isolates were more dominant in all patients it recording 42 isolates (50.6%) while, the isolates appeared as Gram positive reaction reached to 31 (37.34%) isolates and 10 isolates appeared with mixture of both Gram positive and Gram negative with mixture of both cocci and bacilli shapes.

Table (1): Demographic and presence of bacterial isolates in 47 patients with DFI:

Characteristics Age-range (years)	16-35 (6.38%) N = 3				36-55 (44.68%) N = 21				56-75 (49%) N = 23			
Sex	Male		Female		Male		Female		Male		Female	
	N = 1 (33.33%)		N = 2 (66.67%)		N = 9 (42.85%)		N=12 (57.14%)		N=11 (47.82%)		N=12 (52.17%)	
	Macck	Blood	Macck	Blood	Macck	Blood	Macck	Blood	Macck	Blood	Macck	Blood
Growth on medium total	N = 1	N = 0.0	N = 2	N = 2	N = 6	N = 7	N = 11	N = 12	N = 11	N = 11	N = 11	N = 9
Gram-staining	+		-	Mix	+		-	Mix	+		-	Mix
	1		4	0.0	14		20	2	16		21	5
Total isolates	5 (6.02%)				36 (43.37%)				42 (50.60%)			

Susceptibility of Gram-positive isolates to different antibiotics:

Twenty- four isolates that gave Gram-positive reactions were tested for their susceptibilities to 13 different antibiotics; that are routinely prescribed for human treatment by using the disc diffusion method. Results in

Table (2) revealed that, all isolates were sensitive to FOX 30 and IPM 10, while, E15

was resistant by 83.3% followed by SXt 25 (66.7%) and PB30 (54%) of test isolates.

Eight antibiotics about (62%) appeared with resistance by 8-30% of tested bacteria. On the

other hand, isolates B1, B5 and B25 appeared sensitive to all tested antibiotics while, isolates B11, B14 & B16 gave the highest percentage of resistance to antibiotics (46.6%) followed by isolates B30 (38%) and B27a & B36b (30.0%). Also isolates B9, B12, B17, B23, B28, B31b

were moderately resistant to tested antibiotics (23.1%). Other isolates were resistant to one or 2 of the tested antibiotics. The highest inhibition zone (42 mm), recorded with isolate No B12 it produced from around the disc contains 30 ug of AMC.

Table (2): Susceptibility of Gram-positive isolates to different antibiotics.

Isolate No.	Cephalosporins				Polymyxins	Penicillins			Fluoroquinolone	Aminoglycoside		Macrolides	Sulfonamide	% Resistance
	FOX ₃₀	FEP ₃₀	CAZ ₃₀	CRO ₃₀	PB ₃₀₀	PRL ₁₀₀	IPM ₁₀	AMC ₃₀	CIP ₅	AK ₃₀	CN ₁₀	E ₁₅	SXT ₂₅	
B1	20.00	22.00	14.00	19.00	12.00	18.00	27.00	20.00	19.00	20.00	17.00	22.00	26.00	0.00
B2	20.00	10.00	12.00	10.00	0.00	15.00	20.00	12.00	20.00	13.00	8.00	20.00	20.00	7.70
B5	25.00	8.00	10.00	22.00	14.00	10.00	28.00	28.00	10.00	16.00	17.00	15.00	20.00	0.00
B6	23.00	20.00	17.00	15.00	13.00	25.00	21.00	10.00	28.00	18.00	15.00	10.00	25.00	0.00
B7	24.00	15.00	15.00	20.00	0.00	12.00	17.00	10.00	30.00	13.00	15.00	0.00	20.00	7.70
B8	18.00	25.00	17.00	30.00	0.00	20.00	15.00	23.00	30.00	15.00	20.00	0.00	18.0	15.4
B9	12.00	0.00	15.0	20.00	0.00	10.00	10.00	12.00	25.00	18.00	8.00	0.00	0.00	26.70
B10	15.00	12.00	15.00	18.00	15.00	15.00	18.00	14.0	26.00	15.00	10.00	0.00	0.00	15.40
B11	22.00	0.00	0.00	0.00	0.00	10.00	13.00	12.00	0.00	15.00	8.00	0.00	0.00	46.7
B12	12.00	12.00	20.00	30.00	18.00	15.00	25.00	28.00	0.00	20.00	10.00	0.00	0.0	23.1
B14	0.00	0.00	20.00	12.00	12.00	0.00	25.00	18.00	0.00	20.00	11.00	0.00	0.00	46.2
B15	19.00	7.00	0.00	8.00	0.00	0.00	18.00	29.00	8.00	13.00	0.00	0.00	0.00	46.2
B16	10.00	0.00	12.00	12.00	0.00	12.00	16.00	0.00	17.00	12.00	0.00	0.00	0.00	46.2
B17	22.00	30.00	21.00	0.00	0.00	0.00	17.00	30.00	20.00	15.00	7.00	8.00	7.00	23.1
B19	25.00	29.00	30.00	28.00	11.00	35.00	25.00	42.00	0.00	27.00	12.00	17.00	18.00	7.7
B22	18.00	35.00	38.00	31.00	17.00	17.00	21.00	15.00	18.00	18.00	7.00	0.00	0.00	15.4
B23	14.00	35.00	40.00	25.00	0.00	15.00	12.00	14.00	20.00	10.00	10.00	0.00	0.00	23.1
B24	10.00	12.00	14.00	10.00	14.00	15.00	17.00	20.00	12.00	17.00	0.00	15.00	0.00	15.4
B25a	20.00	10.00	10.00	15.00	18.00	25.00	26.00	26.00	20.00	20.00	16.00	12.00	25.00	0.00
B25b	15.00	0.00	0.00	12.00	15.00	20.00	11.00	16.00	0.00	13.00	12.00	15.00	0.00	30.8
B27a	15.00	16.00	15.00	12.00	10.00	0.00	17.00	11.00	18.00	16.00	0.00	0.00	0.00	30.8
B27b	15.00	15.00	10.00	15.00	10.00	12.00	15.00	20.00	17.00	15.00	15.00	0.00	25.00	7.7
B28	15.00	15.00	13.00	15.00	0.00	14.00	12.00	10.00	17.00	12.00	0.00	0.00	11.00	23.1
B30	14.00	0.00	15.00	14.00	12.00	10.00	10.00	0.00	0.00	15.00	20.00	0.00	0.00	38.5
B31a	26.00	13.00	15.00	11.00	15.00	12.00	18.00	17.00	26.00	12.00	12.00	0.00	22.00	7.7
B31b	20.00	0.00	10.00	0.00	10.00	15.00	20.00	15.00	22.00	20.00	17.00	0.00	22.00	23.1
B32a	25.00	0.00	15.00	0.00	0.00	17.00	20.00	15.00	23.00	0.00	0.00	25.00	22.00	38.5
B32b	26.00	11.00	12.00	0.00	0.00	17.00	22.00	15.00	22.00	0.00	0.00	22.00	22.00	30.8
B33	20.00	0.00	0.00	0.00	20.00	0.00	25.00	12.00	0.00	15.00	14.00	0.00	0.00	53.8*
B36a	15.00	18.00	14.00	15.00	0.00	0.00	18.00	15.00	28.00	18.00	11.00	0.00	0.00	30.8
B36b	12.0	15.00	13.00	20.00	10.00	13.00	16.00	12.00	30.00	17.00	10.00	0.00	0.00	15.4
%R	0.0	7.00	4.00	6.00	13.00	6.00	0.00	2.00	7.00	20.00	7.00	20.00	16.00	
	00%	29%	16%	25%	54%	25%	0.00%	8.3%	29%	8.3%	29%	83.3%	66.7%	

AK₃₀: Amikacin, AMC₃₀: Amoxicillin, CAZ₃₀: Ceftazidime, CIP₅: Ciprofloxacin, CN₁₀: Gentamicin, CRO₃₀: Ceftriaxone, E₁₅: Erythromycin, FEP₃₀: Cefepime, FOX₃₀: Cefoxitin, IPM₁₀: Imipenem, PB₃₀₀: Polymyxin, PRL₁₀₀: Piperacillin, SXT₂₅: Trimethoprim-sulphamethoxazol.

Susceptibility of Gram-negative isolates to different antibiotics:

Forty one isolates with Gram-negative reactions were tested against 13 different antibiotics using disc diffusion method. Results in Table (3) showed that, the highest resistant percentage of isolates (75%) were observed when used Macrolides (E15) followed by sulfonamides (SXT25). In addition to that, antibiotics FEP30, CAZ30, CRO30 of

cephalosporine; polymyxins (PB300); penicillins (PRL100); fluoroquinolone (CIP₅) and aminoglycoside CN10 gave a percentage of resistance ranging between 35-45%.; While aminoglycoside AK30; IPM10; AMC30 and FOX3 gave the lowest percentage 7.3%; 9.8%; and 12.2 respectively.

On the other hand, there are 8 (19.51%) isolates (M6, M9a, M9b, M27b, M32b, M33, M36 and B33) gave the highest percentage of resistance to tested antibiotics ranging from 53-77% followed by 19 (46.34%) isolates (M7, M8, M11, M12, M13, M16a, M17b, M18, M22, M24a, M24c, M25a, M25b, M27a, M28, M35, B32b, B32a, B25b & B15) gave moderate percentage of resistant ranged from 30% to 45% and 7 isolates (17.1%) gave a less moderate percentage of resistant ranged from

15% to 25%, While the lowest percentage (7.7%) of resistant was recorded with 5 isolates (12.2%) M5, M16b, M17a, M31 and B31a.

In general the highest diameter of inhibition zone (50 mm) were observed around the disc containing 10 ug of IPM after incubation of plate containing isolate no M17b.

Also Gram-negative bacteria isolates gave a high number of individuals and percentage of resistance above that in Gram-positive bacteria in all categories (height, moderate or lower % of antibiotics resistant).

Index and MIC of antibiotics resistant of selected isolates:

The Sensitivity of selected and identified isolates was tested against 17 different antibiotics by Vitek system. Results in Table (4) revealed that, *Morganella morganii* (M9) and *Proteus mirabilis* (M11) were resistant to Ampicillin; Ampicillin sulbactam and Aztreonam beta-lactams antibiotics; Cefazolin, cefepime and ceftriaxone from cephalosporins; tetracycline and nitrofurantion with index reached to 0.588. *E. coli* gave index = 0.235 while, it resistant to ampicillin and ampicilline/sulbactam from beta-lactams antibiotics. Also, it is resistant to carbapenems antibiotics.

Serratia fonticola appeared sensitive to 15 antibiotics and resist only fluorquinolones. Minimum inhibitory concentration from ampicillin and ampicillin/sulbactam were 32 ug/ml for *Morganella*; *Proteus microbilis* and *E. coli*. *Morganella Morganii* and *Proteus mirabilis* also showed resistant to cefazolin; cefepime, tetracycline and nitrofurantion at MIC ≥ 64 ; 32; $\leq 4.0 < 1$; 4; 128; 0.5 and 16 ug/ml respectively. All tested bacteria were resistant

also to ciprofloxacin and moxifloxacin at MIC 4 or 8 ug/ml respectively.

Selection and Identification of antibiotics resistant isolates:

The biochemical characteristics of these isolates were determined by Vitek system. According to Bergey's manual (2005) as *Morganella morganii* (M9), *Proteus mirabilis* (M11); *Serratia fonticola* (M16) and *E. coli* (M32).

Effect of plant extracts:

Three traditional medicinal plants (*Rosmarinus officinalis*, *Syzygium aromaticum* and *Ginger officinales*) were obtained from markets and extracted by ethyl alcohol. Each extract was tested against the MDR *Morganella morganii* or *Proteus merabilis* using the well diffusion method. Results in Table (5) and Fig. (1) showed that, *Rosmarinus afficinales* and *Syzygium aromaticum* gave an inhibitory activity (20-27 mm of inhibition zone) against both tested bacteria. The antibacterial activity of *Rosmarinus afficinales* was higher than that of *Syzygium aromaticum*. While, *Zingiber afficinales* not gave any activity. The MIC of *Rosmarinus* reached to 78.13 ug/ml against both bacteria while, MIC of *Syzygium aromaticum* reached to 12.89 ug/ml and 6.45 ug/ml for *Morganella morganii* and *Proteus merabilis* respectively.

Results also revealed that, the highest yield extract (3.39) was obtained from *Syzygium aromaticum* (clove) followed by 2.5 & 1.2 g for *Rosmarinus afficialies* and *Zingiber afficialis* respectively.

Due to that *Syzygium aromaticum* extract was selected for further stud

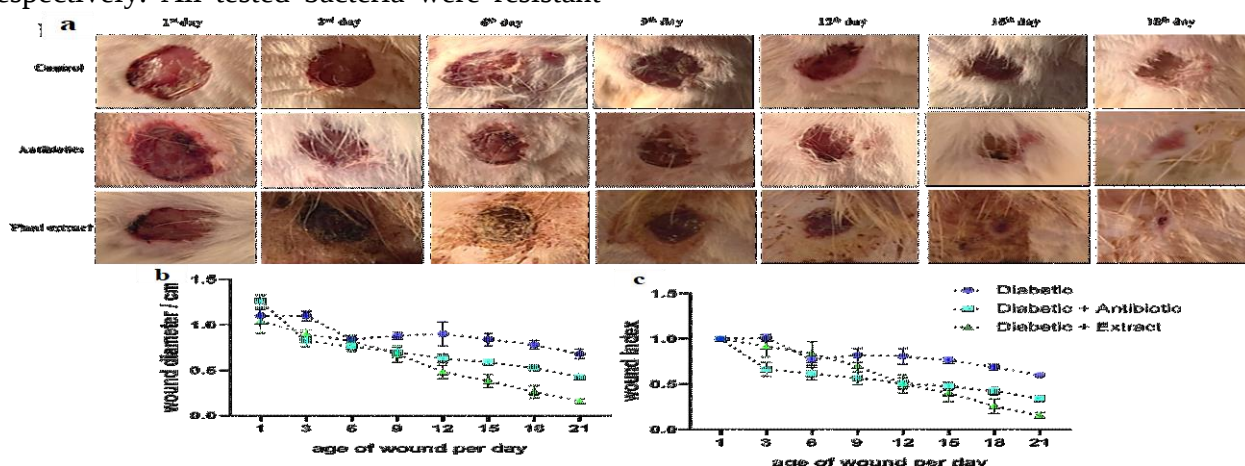


Figure (1): Effect of the hydrogel topical application of Cefepime 20 % and ethanolic clove extract 1.3 % once daily for 14 successive days on the mean value of diabetic wound diameters (cm) (a & b) and wound

index (c) of the diabetic wound infected with clinical isolates of *Proteus Mirabilis* LC587231 in type1 diabetic rats wound imaging, wound diameter and wound index were followed upon 1, 3rd, 6th, 9th, 12th, 15th, 18th and 21st days post-treatment. Values are mean of 8 rats per group $\pm$ SEM. Means were significantly different at $P < 0.05$

Table (3): Susceptibility of Gram-negative isolates to different antibiotics.

Isolate No.	Cephalosporins				Polymyxins	Penicillins			Fluoroquinolone	Aminoglycoside		Macrolides	Sulfonamide	% Resistance
	FOX ₃₀	FEP ₃₀	CAZ ₃₀	CRO ₃₀	PB ₃₀₀	PRL ₁₀₀	IPM ₁₀	AMC ₃₀	CIP ₅	AK ₃₀	CN ₁₀	E ₁₅	SXT ₂₅	
M5	17.00	12.00	10.00	13.00	10.00	20.00	20.00	13.00	25.00	18.00	15.00	0.00	26.00	7.7
M6	0.00	0.00	0.00	0.00	12.00	0.00	10.00	0.00	0.00	15.00	0.00	0.00	0.00	76.9
M7	15.00	18.00	10.00	0.00	8.00	12.00	0.00	0.00	15.00	10.00	15.00	0.00	0.00	38.5
M8	15.00	0.00	15.00	0.00	18.00	10.00	7.00	0.00	25.00	15.00	0.00	12.00	0.00	38.5
M9a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	15.00	12.00	20.00	15.00	0.00	12.00	61.5
M9b	11.00	0.00	0.00	0.00	0.00	0.00	11.00	8.00	16.00	0.00	0.00	0.00	0.00	69.2
M11	20.00	0.00	7.00	11.00	11.00	21.00	22.00	12.00	0.00	13.00	9.00	0.00	0.00	30.8
M12	17.00	0.00	0.00	12.00	0.00	13.00	8.00	14.00	4.00	15.00	0.00	0.00	0.00	46.2
M13	0.0	18.00	17.00	40.00	12.00	20.00	16.00	40.00	0.00	16.00	12.00	0.00	0.00	30.8
M15a	18.00	12.00	10.00	18.00	11.00	16.00	15.00	15.00	20.00	18.00	15.00	0.00	0.00	15.4
M15b	22.00	23.00	18.00	12.00	0.00	15.00	23.00	17.00	36.00	21.00	13.00	0.00	0.00	23.1
M16a	18.00	12.00	0.00	9.00	0.00	20.00	12.00	17.00	0.00	12.00	7.00	0.00	8.00	30.8
M16b	21.00	15.00	15.00	6.00	0.00	24.00	20.00	28.00	7.00	17.00	9.00	7.00	4.00	7.7
M17a	28.00	20.00	19.00	36.00	40.00	0.00	18.00	38.00	28.00	18.00	9.00	6.00	8.00	7.7
M17b	19.00	18.00	30.00	34.00	0.00	30.00	35.00	50.00	19.00	16.00	0.00	0.00	0.00	30.8
M18	15.00	0.00	0.00	8.00	0.00	15.00	11.00	12.00	15.00	0.00	16.00	0.00	10.00	38.5
M22	0.00	0.00	17.00	19.00	0.00	0.00	21.00	10.00	15.00	17.00	9.00	0.00	5.00	38.5
M23	10.00	10.00	15.00	8.00	0.00	9.00	18.00	17.00	25.00	15.00	7.00	0.00	0.00	23.1
M24a	20.00	15.00	15.00	14.00	0.00	15.00	10.00	12.00	11.00	16.00	0.00	0.00	0.00	30.8
M24b	15.00	28.00	17.00	15.00	20.00	13.00	20.00	10.00	12.00	15.00	7.00	0.00	0.00	15.4
M24c	18.00	19.00	14.00	15.00	0.00	10.00	12.00	13.00	12.00	15.00	0.00	0.00	0.00	30.8
M25a	17.00	0.00	0.00	0.00	12.00	0.00	23.00	11.00	0.00	14.00	13.00	12.00	16.00	38.5
M25b	17.00	0.00	0.00	10.00	11.00	0.00	22.00	11.00	0.00	18.00	17.00	14.0	12.00	30.8
M27a	17.00	0.00	15.00	0.00	0.00	8.00	17.00	10.00	0.00	15.00	0.00	0.00	22.00	46.2
M27b	19.00	0.00	0.00	0.00	0.00	11.00	15.00	8.00	0.00	15.00	0.00	0.00	0.00	61.5
M28	15.00	15.00	17.00	18.00	0.00	15.00	16.0	12.00	17.00	12.00	0.00	0.00	0.00	30.8
M30	25.00	22.00	17.00	20.00	14.00	15.00	32.00	25.00	25.00	22.00	20.0	0.0	0.00	15.4
M31	20.00	22.00	15.00	20.00	10.00	9.00	31.00	16.00	22.00	16.00	18.00	0.00	16.00	7.7
M32a	13.00	0.00	7.00	15.00	44.00	0.00	13.00	10.00	30.00	12.00	11.00	0.00	7.00	23.1
M32b	9.00	8.00	0.00	0.00	19.00	0.00	0.00	10.00	0.00	0.00	17.00	0.00	0.00	61.5
M33	0.00	7.00	12.00	0.00	18.00	0.00	29.00	10.00	0.00	16.00	0.00	0.00	0.00	53.9
M35	15.00	25.00	18.00	31.00	0.00	0.00	17.00	0.00	18.00	15.00	19.00	0.00	0.00	38.5
M36	18.00	7.00	0.00	0.00	11.00	0.00	0.00	0.00	0.00	15.00	0.00	0.00	0.00	69.2
R%	12.2	36.6	34.14	34.14	46.30	34.14	9.80	12.2	31.7	7.31	39.0	75.60	61.01	

Discussion:

According to the centers for disease control and prevention (CDC), Egypt is among 10 top countries with the highest prevalence of diabetes and ranked ninth in the world, where there are 7 million and a half million Egyptians are living with diabetes and up to 15% of those with diabetes will develop a foot ulcer during their lifetime [23].

Diabetic foot ulcers (DFU) are most common in diabetic patients and frequently 40-80% of these patients are infected [24]. Most severe infection are usually polymicrobial and several bacterial genera can be part of their microbiota particularly aerobic Gram-positive cocci such *S. aureus*, *Staphylococcus epidermidis* and *Enterobacter* spp., Gram-negative bacilli such as *Pseudomonas* spp.,

Escherichia coli, *Enterobacter* spp., *Acinetobacter baumannii* spp. And *Citrobacter* spp. And anaerobes such as *Bacteriodes* spp., *Peptostreptococcus* spp., *Fusobacterium* spp. and *Clostridium* spp. [25,26,27,28].

In this study it was found from 47 patients with diabetic foot infection and 83 bacterial isolates were collected from positive cultures cases. All cultures contain polymicrobes. These results are agreements with that found by Jneid et al. [2]. They reported that most of their samples were also polymicrobial. Our isolated appeared with dominant of bacilli Gram-negative bacteria. It reached to 50.60% while Gram-positive isolates reached to 37.34% and about 12% of cultures contain cells of Gram-positive and Gram-negative. These results are similar with that founded by Jnana et al. [10]; Sanchez-Sanchez et al. [29]; Shanmugam &

Jeya [30] and Turhan et al. [31]. They reported that Gram-negative bacilli were more prevalent than Gram-positive cocci.

The geographical origin of the patient seems to be one of the most important factors. Indeed, in warmer countries (particularly in Asia and Africa), Gram-negative bacilli are more prevalent compared to western countries [32,33].

On the other hand, Citron et al. [8] reported that aerobic gram-positive cocci are the predominant organisms responsible for acute DFUs, *Staphylococcus aureus* is the most commonly isolated pathogen; while in chronic wounds, the most predominant bacteria are Gram-negative bacilli and obligate anaerobic bacteria [34].

In recent years, the emergence of antibiotic-resistant pathogens made it increasingly difficult to select appropriate empirical antibiotics for the treatment of DFI [35].

There is a paucity of data on multi-antibiotics resistant bacteria isolated from diabetic foot infections [26]. Shanmugan et al. [30] showed 37.5% of the Gram-negative bacilli were ESBL producers and 31% were carbapenemase producer.

Due to that this study aimed to study the bacterial profiles of diabetic foot ulcer and determine the susceptibility of these bacteria to different antibiotics. Results revealed that by 8-30% of our isolates were G +ve with resistance to 62% of tested antibiotics. Macrolide (E15) recorded the highest percentage of resistant (82%) followed by sulfonamide (66.7%), meanwhile results obtained by Costa et al. [35] showed that 23.3% (n=25) were MDR. The increasing percentage of resistance in tested Gram positive bacteria may be to increase the % of MRS bacteria. Isolates were sensitive to IPM₁₀ (100) and FOX₁₀ % while sensitivity reached to 91.7% when used ampicilin and amikacin. These results are in accordance with that reported by Mathangi and Prabhakaran [36] they showed that amikacin, amoxicillin, chloromycetin, chloromphenicol, levofloxacin and penicillin with good sensitivity against used MRS.

Also macrolides (E15) gave the highest percentage of resistant for Gram-positive (83.3%) and Gram-negative (75.6%) while it

appeared with percentage of resistant reached to 65% in study of Sanchez-Sanchez et al. [29] of their Gram-positive bacteria isolated from DFU in the northeast of Tampaulipas, Mexico.

Our results indicated that aminoglycoside (Amikacin and IPM₁₀) were the most effective antibacterial agents for Gram-negative bacilli where, sensitivity of these antibiotics reached to 92.69% and 90.2% respectively. These results are in harmony with that obtained by Sanchez-Sanchez et al. [29]. Coinciding with our study, Citron et al. [8] reported that the Enterobacteriaceae group was largely susceptible to imipenem and aminoglycosides.

Many organisms showed multidrug resistance. This increasing incidence of multidrug resistant organisms is a potential risk factor in management of diabetic foot infections which may lead to devastating complications like systemic toxicity, gangrene formation and amputation of the lower extremity [37].

Our results revealed that, there are 19.51% of isolates gave a highest percentage of resistant (53-77%) followed by 46.34% of isolates with moderate percentage of resistant (30-45%). Selected isolate (M9 & M11) from high resistant were identified as *P. mirabilis*. Sensitivity of these isolates to different antibiotics were confirmed again. These isolates gave the highest MAR Index: 0.8. Previous studies showed that the occurrence of proteus species is low or moderate in tested infected diabetic foot ulcer, it appeared with high percentage of resistant to antibiotics [10,29].

Saltoglu et al. [38] detected *Proteus* spp. with percentage reached to 32% of diabetic foot infection in multicenter in Turkey. These isolates were appeared with + ESBL.

Our selected isolates M9 & M11 identified as *Morganella morganii* and *Proteus mirabilis* by vitacks being similar to that isolated by Mathangi and Prabhakaran [36] where MDR index of their isolates reached to 0.8 and 0.6 respectively. From internestic antibiotics resistance there are high similarity between both isolates M9 & M11. Isolated M9 (*Morganella morganii*) appeared resistant to sulfonamidis, while isolate M11 (*Proteus mirabilis*) appeared sensitive with MIC reached

to ≤ 20 ug/ml. O'Hara et al. [39] considered the intrinsic resistance to tetracycline as identification markers for *P. mirabilis*. From these results two isolates may be *P. mirabilis* to confirm these results, identification by 16s was carried. Results of identification by 16s revealed that isolate no 9. Identified as *Proteus mirabilis* but have mutant in gen.

Wang et al.[40] showed a significant decrease in susceptibility to 4th generation cephalosporins and ciprofloxacin occurred in *P. Mirabilis* from Taiwan in the past decades (2002-2012).

They attributed these finding to increase production of *P. mirabilis* produced Ampc β -lactamase while, ESBL remained stable. Due to that the minimum inhibitory concentration (MIC) and the dosing regimen must be carefully evaluated before treatment to insure effective coverage [41]. Due to that Patil et al. [41] concluded that cefepime should be avoided for empiric treatment of suspected ESBL infections and should only be considered for definitive treatment if the MIC ≤ 1 ug/ml. Addition of cefepime at MIC = 1 to nutrient agar media and pured in sterilized plates. This concentration not suitable to inhibit the growth of *P. mirabilis*-isolate No. 11. The isolated *P. mirabilis* (M9 & M11) inhibited only at 25 ug/ml and 100 ug/ml for both isolates respectively.

From above it should be screened for another natural product to control the isolated MAR *P. mirabilis* (isolates M9 M 11).

Microbial resistance to antibiotics and its rapid progression has raised serious concern in the treatment of infectious diseases [42]. Recently, many studies have been directed towards finding promising solutions to overcome these problem. Phytochemicals and probiotics have exerted potential activities against MDR bacteria [9,42,43]. Our results showed that the ethanolic extracts of *Rosmarinus officinalis*, *Syzygium aromaticum* and *Zingiber officinalis* gave antibacterial activity against MDR *P. mirabilis* isolates M(9 & 11). Clove gave the highest inhibitory effect against both proteus isolates with lowest MIC.

These results are similar to that obtained by Sarhan et al. [44] they isolated 7 isolates of

MDR *P. mirabilis* from infected diabetic foot ulcer of patient in Mansoura, Egypt Specialized Medical Hospital. Clove extract gave 2.

Jneid, J.; Lavigne, J.P.; LaScola, B. and Cassir, N. (2017): The diabetic foot microbiota. Human Microbiome Journal, 5-6: 1-6.

antibacterial activity against all tested MDR *P. mirabilis* isolates, while, these isolates were resistant to 60% of tested plant extracts. Also studies of Kozics et al. [45] showed that clove extract gave MIC and MBC = 0.05 w/v against MDR isolate of *P. mirabilis* KMB522.

Extract of *Syzygium aromaticum* (Clove) recorded by Khameneh et al. [42] as strongest plant antibacterial. This plant contain eugenol which active against several microorganisms. Eugene have ability to disturbance the composition of plasma membrane. Destruction of external membrane, cytoplasmic membrane and energy metabolism of cells can cause the loss of permeability, leakage of intracellular constituents and even the coagulation of cytoplasm [46] and Rathinam et al. [47] showed Eurganol inhibit the biofilm formation and virulence factor synthesis of *P. aeruginosa*.

Several types of proteins were responsible for wound healing as; MMP3, MMP9, collagen, and fibronectin that secreted from both keratinocyte and fibroblast [48], The result of the present investigation revealed that the topical application of *Syzygium aromaticum* extract elicited a significant improvement in wound size and wound index in comparison to both antibiotic-treated group and control one that follows [49].

Application of the *Syzygium aromaticum* extract hydrogel induced a marked upregulation in the relative expression of the growth factor related to both angiogeneses, keratinocytes, fibroblast growth, and proliferation that could be attributed to the increased expression of the glucagon like peptides and their receptors thus potentiating secretion of the growth factors such as; vascular endothelial growth factors (VEGF) that improved wound microcirculation [50], epidermal growth factor (EGF)[51] and fibroblast growth factors[52] that strengthened the growth and proliferation of the keratinocytes and fibroblast [53].

Table (4): Index and MIC of antibiotics resistance of selected isolates.

M32	M16	M11	M9	Isolate No		
				Identified as:		
<i>Escherichia coli</i>	ND	>=32R	>=32R	Ampicillin	1- Beta-lactams	
	ND	>=32R	>=32R	Ampicillin/sulbactam		
	<=1.0S	<=1.0*R	>=64R	Aztreonam	Mono-bactam	
	<=0.5S	<=0.5S	<=0.5S	Ertapenem	Carbapenems	
	<=0.25S	<=0.25S	=2.0I	Imipenem		
	<=0.25S	<=0.25S	<=0.25S	Meropenem		
	<=4.0S	<=4.0*R	>=64R	Cefazolin	2- Cephalosporins	
	<=1.0S	<=1.0*R	=32R	Cefepime		
	<=1.0S	<=1.0*R	=4.0R	Ceftriaxone		
	<=2.0S	<=2.0S	<=2.0S	Amikacin	Aminoglycosides	
	<=1.0S	<=1.0S	=8.0I	Gentamicin		
	<=1.0S	<=1.0S	<=1.0S	Tobramycin		
	>=4.0R	>=4.0R	=2.0I	Ciprofloxacin	Fluoroquinolones	
	>=8.0R	>=8.0R	>=8.0R	Moxifloxacin		
	<=0.5S	<=0.5*R	=4.0*R	Tetracyclines (Tigecycline)		
	<=16S	<=16*R	=128R	Nitrofurantion		
	<=20S	<=20S	>=320R	Sulfonamides		
				Trimethoprim sulfamethoxazole		

Table (5): Susceptibility of isolated bacteria to different medicinal plant extracts.

Botanical name	General name	Yield (g)	<i>Morganella morganii</i> (M9)	<i>Proteus mirabilis</i> (M11)
<i>Rosmarinus officinalis</i>	Rosemary	2.5	23.00MIC = 78.13 ug/ml	27.00MIC = 78.13 ug/ml
<i>Syzygium aromaticum</i>	Clove	3.3	20.00MIC = 12.89 ug/ml	25.00MIC = 6.45 ug/ml
<i>Ginger officinales</i>	Ginger	1.2	0.00	0.00

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