Inhibitory Effect of *Myrtus communis L.* and *Syzygium aromaticum L.* Extracts on the Growth of *Staphylococcus aureus* Isolated from Foot Ulcers of Diabetic Patients

Mohammed Khalefa Khadir[1], Abbas Yaseen Hasan[2] and Rana Mohammed Salman[3]

Department of Biology - College of Science - University of Diyala - Diyala - Iraq

[3]salmsamar226@gmail.com

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Abstract

The study included 104 of two types of diabetic patients with foot ulcers. both male and female are involved. their age ranged (35-74) years, the first type of diabetes mellitus is insulin dependent and the second type is insulin nondependent. The results showed a significant difference ($P<0.05$) in infected males 75.96% compared with females 24.04%. The study has been performed at Baquba Teaching Hospital during the period from October 2016 to the end of March 2017. In this study, 35 isolates of *Staphylococcus aureus* have been isolated and diagnosed according to bacteriological and biochemical diagnostic criteria. The study showed that the hot aqueous extract of *Myrtus communis* and *Syzygium aromaticum* had strong effect at concentrations of (12.5,25,50,100,200 mg/ml) against the selected isolate of *S. aureus* with the diameter of inhibition zone (10.20,14.00,16.33,19.21, 22.27mm. respectively) for the first plant, while they were (9.80,12.31,15.56,19.21,21.22 mm. respectively) for the second plant. At the same concentrations mentioned above. the alcohol extract showed an inhibitory effect and the diameter of the inhibition zones were (8.23,12.31,14.21,18.33,20.31mm. respectively) for the first plant, while they were (13.00, 16.33,18.33 ,20.31,24.04mm. respectively) for the second plant. In comparison, the alcohol extract of *Syzygium aromaticum* showed the higher inhibitory activity against *S. aureus*. Antibiotic sensitivity performed on all bacterial isolates.

twelve type of antibiotics were used, the isolates of bacteria revealed 100% sensitivity to both Vancomycin and Rifampin, while 100% resistance showed to Ampicillin and Penicillin G.

Key words: Diabetes mellitus, Foot ulcers, *Staphylococcus aureus.*

التأثير التثبيطي لمستخلصات نباتي الآس ***Myrtus communis L.*** والقرنفل ***Syzygium aromaticum L.*** ضد نمو بكتريا ***Staphylococcus aureus*** المعزولة من قرحة اقدام مرضى السكري .

محمد خليفة خضير، عباس ياسين حسن و رنا محمد سلمان

قسم علوم الحياة - كلية العلوم - جامعة ديالى - ديالى - العراق

الخلاصة

شملت الدراسة 104 مريضاً مصابً بتقرحات القدم السكري من الذكور والاناث تراوحت اعمارهم بين(35– 74) سنة من مرضى داء السكري النوع الاول داء السكري المعتمد على الانسولين و النوع الثاني غير المعتمد على الانسولين . اذ اظهرت النتائج فرقاً معنويـــاً ($p< 0.05$) لدى الذكور المصابين وبنسبة 75.96% مقارنة مع الاناث بنسبة 24.04%.أجريت الدراسة في مستشفى بعقوبة التعليمي خلال الفترة من أكتوبر 2016 حتى نهاية مارس 2017. في هذه الدراسة تم عزل 35 عزلة لبكتريا *Staphylococcus aureus* وتم تشخيصها اعتماداً على الصفات البكتريولوجية والكيموحيوية القياسية. وأظهرت الدراسة أن المستخلص المائي الحار لنباتي الآس*Myrtus communis L.* والقرنفل*Syzygium aromaticum L.* تأثيرا واسعاً بالتراكيز (200,100,50,25,12.5) ملغم/مل ضد عزلات بكتريا *S. aureus* باقطار تثبيط(14.00,10.20 22.27,19.21,16.33ملم على التوالي) للنبات الأول، في حين كانت (21.22،19.21،15.56،21.31،9.80 ملم على التوالي) للنبات الثاني. وبنفس التراكيز اعلاه, وكذـلك اظهر المستخلص الكـحولي تاثير تثبيطي وبأقطار تثبيط (20.31,18.33,14.21,12.31,8.23 ملم على التوالي) للنبات الاول. في حين كانت (24.04, ,20.31,18.33,16.33,13.00 ملـــــــــم على التوالي) للنبات الثاني. وعند المقارنة اظهر المستخلص الكحولي لنبات القرنفل اعلى مناطق تثبيط ضد بكتريا*S. aureus*. اجريت حساسية المضادات الحياتية على جميع العزلات البكتيرية تجاه اثنا عشرنوعاً من المضادات الحياتية المستخدمة في الدراسة, اذ ا ابدت عزلات البكتريا تحسسا 100% تجاه مضادي Vancomycin و Rifampin , في حين اظهرت مقاومة 100% لمضادي Ampicillin و Penicillin G .

الكلمات المفتاحية: داء السكري، قرحة القدم، المكورات العنقودية الذهبية .

Introduction

Diabetes refers to the disorder of the endocrine glands that affect the Beta – cells of the Langerhans Islands Which lead to insulin deficiency in the blood, More than 700000 people Worldwide are diagnosed with this disease each year which affects both sexes and all age groups [1]. Diabetic patients have serious complication of foot ulcers leading to patients being admitted to hospitals. The ulcers become contagious and may be develop in the skin, muscle and foot bones later due to peripheral nerve damage and poor blood circulations in the area of infection causing a disease called Gangrene. which requires an amputation of the lower limb. Diabetic foot wounds are the major cause of mortality of patients with diabetes. The severity of the infected wound range from moderate to severe and the period of healing no less than six month. about (1- 3) % of the cases undergo an amputation [2]. *Staphylococcus aureus* naturally found on the skin and mucus membrane of human body at (30 – 35) % [3], colonizes the nose and the skin. It is part of normal flora but may causes serious infections such as Rheumatoid arthritis, Toxic Lens syndrome, Endocarditis, Meningitis, Pneumonia and congenital heart valve inflammation [4]. Bacteria causes a large Number of human infections worldwide ranging from mild skin injuries and severe such as deadly Rheumatoid arthritis and necrotizing fascia, as well as inflammation of bloodstream leading to manifestation of severe disease such as Sepsis and Deep cysts of Dental roots [3]. The bacteria is the main causes of diabetic foot infections. A study conducted by Lela *et al*. [5]in Egypt on diabetic foot patients has shown that most of the microorganisms that diagnosed from foot swabs were due to *S. aureus* which at 20 cases infected by this bacteria out of 59 cases. Pathogenesis of bacteria for diabetic foot ulcers classification according to physiological of skin composition and the infected soft tissue when bacteria enter the infected skin the Neutrophils and Macrophage migrate to the injured area, bacteria pass though define line in different ways, including blocking the antibody and chemical attraction of white blood cells by forming capsule and biofilm thus resisting the crash after ingestion by phagocytes [6], in addition to the mechanism of the adhesion. bacteria has the ability to produce toxins as their virulence factors contributing to the inflammation of foot which plays an important role in colonization. dissemination and persistence [7]. Recent research indicated that the effectiveness of medical plants and their secondary compounds as

effective antimicrobial agents, this concern the result frequent and indiscriminate use of antibiotic, which led to the emergence of high resistance to industrial antibiotics when taken for long periods and the side effects, encouraged researchers and many people to use natural plants extracts as therapeutic material [8].one of the plants used in the study was *Myrtus communis* (myrtle) an evergreen tree hight up to (1-5) m [9]. It belongs to the Myrtaceae family and grows spontaneously in all areas of the Mediterranean sea. It is an aromatic plant because of it's high content of oils for leaves, flowers and fruits [10]. *Myrtus communis* extract has been used as a sterilizer for wounds and infected skin, and it had a great medical importance in the treatment of Diarrhea. Stomach ulcer, Hemorrhoids, Bleeding and Coughing [11]. *Syzygium aromaticum* (Clove) is a long –standing tree planted in gardens, has a medium-size hight up to (8-12) m. It grows in Malaco Islands of Eastern Indonesia, *Syzygium aromaticum* is one of the most important plants in the world. it an evergreen trees with a conical shape that has a smart smell and dark green leaves. The seeds are red in color like nails and they are widely used [12, 13]. *Syzygium aromaticum* extract has great efficacy in treating many of the infections. It has been used in the treatment of the abdominal pain. Asthma and Allergies. Also used as a disinfectant for Wounds, Ulcers and in the treatment of painful teeth. Dyspepsia, Stimulant for the heart and as an anti –Vomiting [14]. The study aims to diagnose *S. aureus* isolated from diabetic foot ulcer and to show the inhibitory effect of *Myrtus communis L.* and *Syzygium aromaticum L.* against this bacteria

Material and Methods

Bacterial isolates

Study was conducted at the beginning of October 2016 to the end of March 2017. 104 swab samples were collected from patient's foot ulcers at Baquba Teaching Hospital for both sexes and with ages ranged (35- 74) years from different areas of the ulcer foot. The samples cultured directly on Blood Agar at 37^{o}C for 24 hours, the single colonies were then elected and cultured on Mannitol Salt Agar. Biochemical and Diagnostic tests were used for diagnosed *S. aureus* depending on what reported in MacFadden [15].

Collection of plant specimens

The Myrtle leaves collected from public nurseries in the city of Baquba. The Seeds of *Syzygium aromaticum* were taken from the herb offices in Diyala Governorate. The plants were left in the laboratory for one week at 25ºC for drying. An electric obtain a powder that was kept in dark glass bottles.

Preparation of the aqueous extract

Parekh and Chanda methods were used [16] with weight of 50 gram of dry plant powder, added to 500 ml of boiling distilled water. it was left for 30 minutes in shaking incubator at 37ºC.The extracts was filtered by Whatman filter paper 1,3,6 centrifugations was done at 2500 rpm speed for 10 minutes, and the remaining spray evaporation by using Rotary evaporator under low pressure at (40–50)ºC, by using Oven at 40ºC, The process was repeated several times to obtain sufficient amount of extract. The extract powder was packed in sterile dark bottle and refrigerator at 4ºC until use.

Alcoholic extract: the of Preparation

Fifty gram of dry plant powder were added to 500 ml of ethyl alcohol at 70%. left in shaking incubator for 24 hours at 30ºC. the extract was than filtered by filtration papers, and then centrifugation was done at speed of 2500 rpm for 10 minutes. than display the remaining spray evaporation by using Rotary evaporator under low pressure at (40 –50) º C, the remaining was dryed by using an oven at 40ºC. The process was repeated several time to obtain a sufficient amount of extract powder. The extract powder was packed in sterile dark bottle and refrigerator at 4ºC until use [17]

Effect of *Myrtus communis L.* and *Syzygium aromaticum L.* on bacterial growth

Well Diffusion Assay method was used according to what explained by Obaidte *et al*.. [18] The bacterial suspension was prepared by taking a number of bacterial colonies by loop and placing them in Brain Heart Infusion Broth (BHIB) to activate the bacteria. The tubes were incubated for 18 hours at 37ºC and the bacterial suspension was compared with the McFarland solution by counting the number of bacteria (1.5×10^8cell/ ml). By using sterilized swab, he bacterial

suspension was spread on Muller Hinton Agar in several directions. The medium was left to dry and than 5 mm wells were made by using cork borer. A series of concentrations from an aqueous and alcohol extracts were per-prepared (200, 100, 50, 25, 12.5mg/ml) and 0.1 ml of the above concentrations were added to each well sequentially. For comparison, controls were used by replacing the aqueous extracts and the alcoholic extracts with distilled water and buffer phosphate solution (PBS), respectively. were applied for each plate, after then all plates incubated at 37ºC for 24 hours. To determine the effectiveness of each concentration of the selected extract. diameter of inhibition zone in mm was measured by using a standard ruler.

Antibiotic sensitivity test

Antibiotic sensitivity test was conducted as reported by Morello *et al* [19], where Disk diffusion method was used to test the bacterial sensitivity to antibiotics using Muller- Hinton agar and then (3-5) bacterial colonies were transferred by loop to tube containing 5 ml of Nutrient broth and then incubated at 37ºC for 24 hours, About 0.1 ml liquid bacterial growth was transferred to tube containing normal saline, the growth turbidity solution was compared to the standard turbidity solution which gives approximately (1.5×10^8cell/ ml), then 100 µl of bacterial suspension transfer by micro pipette, and diffused by the glass diffuser on Muller- Hinton agar surface. The plates were left to dry at room temperature for (10-15) minutes. Six to seven antibiotic disks were used per plate then the plates were incubated at 37ºC for 24 hours. Each antibiotic disk was measured based on CLSI [20]. The antibiotics used in this study were: Amoxicillin / Clavulanic acid (20/10 µg), Ampicillin (10 µg), Cefotaxime (30µg), Ciprofloxacin (5µg), Erthromycin (15µg), Gentamycin (10µg), Imipenem (10µg), Ofloxacin (5µg), Oxacillin (1µg), Penicillin (10IU), Rifampin (10µg) and Vancomycin µg) **(**10 µg)

Result and Discussion

The results of the current study (Table1) showed that 79 men (75.96%) and 25 women (24.04%) were found infected with diabetic foot ulcer and the difference between the two gender was significant (P<0.05), The results was consistent of the Esmat and Saif- Al Islam [21] and Reghu *et al*. [22]. This may be due to the difference in physical strength between men and women and

weakness of man's joint and increased pressure on them. lack of sensitivity of men to the deterioration of nerve in the affected area of the foot [23].

Table 1: Distribution of diabetic foot ulcers patients by gender

Gender	Number	Percentage (%)
Male	79	75.96%*
Female	25	24.04%
Total	104	100%

* ($P<0.05$), Chi square test

During this study. 35 bacterial isolates of *S. aureus* were diagnosed depending of biochemical test and this bacterium is important and dangerous pathogen causing different types of infections and the most important in clinical terms. Its pathogenicity the ability to produce many virulence factors which include toxins such as Heamolysin which works on the breakdown of red blood cells and platelets. toxic to some cells and tissues and formation of biofilm. which gives the bacteria the ability to multiply and spread within host tissues. as well as its high resistance to multiple antibiotics especially those belong to the group Beta-lactam and Aminoglycosides [24]. Table (2) shows that the aqueous extracts of the Myrtle was more effective at the highest concentration of 200 mg/ml as judged by the diameter of the inhibition zone which was 22.27mm. followed by the concentrations (100,50,25 mg/ml) with inhibition zone of (19.21,16.33 and14.00mm. respectively). In contrast the lowest effect was at the concentration 12.5 mg /ml and the diameter of inhibition zone was 10.20 mm. The differences were significant the diameter of the inhibition zones of the different concentrations. This result was consistent with the study of Ali *et al*. [25] who investigated the inhibitory ability of the aqueous extract of leaves of the Myrtle on the growth of *S. aureus.* and their that the alcoholic extracts had inhibitory effect against *S. aureus* as evidenced by the diameter of the inhibition zone which was 20.31 mm at concentration 200 mg/ml. followed by the concentration (100,50,25mg/ml) and the diameter of the inhibitory (18.33,14.21, and 12.31mm. respectively), while the diameter of the inhibition zone was 8.23 mm at the concentration of 12.5 mg/ml (Table 2). The results were consistent with the Taheri *et al* [26]and Pandy and Singh [27] who demonstrated the inhibitory efficacy of alcoholic extract of Myrtle against *S. aureus*.

Table 2: Effect of aqueous and alcoholic extracts of *Myrtus communis* on *S. aureus*.

(Concentration mg/ml)	Aqueous extract Diameters of the inhibition zone (mm)	Alcoholic extract Diameters of the inhibition zone (mm)
	Mean ± St. Error	Mean ± St. Error
0.00	0.00± 0.00	0.00± 0.00
12.5	0.34 ± 10.20	0.33 ± 8.23
25	0.25 ± 14.00	0.55 ± 12.31
50	0.76 ± 16.33	0.41 ± 14.21
100	0.23 ± 19.21	0.36 ±18.33
200	0.40 ± 22.27	0.55 ± 20.31

($p<0.05$), Duncan test (Mean ± St. Error)

Study of Aleksic and Knezeric [28], indicated the inhibition role of the aqueous and alcoholic extracts of Myrtle against Gram positive and Gram-negative bacteria. Aidi-Wannes *et al*. [29]have interpreted the antimicrobial activity of the Myrtle against bacterial strains that may be due to the presence of active compounds in the leaves which were of three main groups: phenolic acids (such as Valic acid, Garlic acid, Cafeic acid and Ellagic acid), tannins (Proanthociandidins tannins, Gallo tannins and Hydrosable tannins), and flavonoids (Gurecetin, Catechin and Myricetin). These compounds have effective and inhibitory effect on growth of Gram positive and Gram-negative bacteria. A comparison between the effect of the aqueous and alcoholic extract of the Myrtle revealed significant differences ($P < 0.05$) in favor of the aqueous extracts may be because the hot water has enhance the extraction of active substance such as turbines, phenols and alkaloids that have inhibitory activity against microorganism. The study of Genetu *et al*. [30] showed that the efficacy of aqueous extracts from the leaves of Myrtle plant were more effective against the bacteria than the alcoholic extracts. The efficacy of the Myrtle leaves extracts was attributed to their contents of highly toxic phenols and poly phenols that inhibit growth of Gram positive and negative bacteria. as well as the presence of the other compounds such as α- phinene and 1.4 cineole which are effective in inhibiting growth of bacteria [29] The current study showed that the aqueous extract of *S. aromaticum* antibacterial effect against *S. aureus*. evidenced by the diameter of the inhibition zone which was 21.22mm at concentration 200mg/ml. followed by the concentrations (100, 50, 25, 12.5 mg/ml) and the diameter of the inhibition zone were (19.21, 15.56, 12.31, 9.80 mm.

respectively), (Table 3). The result of the study conducted by Al-Dhaher [31] which showed effectiveness of aqueous extract of *S. aromaticum* against *S. aureus.* In addition the of the present that the alcoholic extract of *S. aromaticum* had inhibitory effect against *S. aureus* at concentrations of (200, 100, 50, 25, and12.5 mg/ml) and the results are shown in Table 3. Similarly the study of Mourad *et al*. [32] also showed the effect of alcoholic extract of *S. aromaticum* against *S. aureus*

Table 3: Effect of aqueous and alcoholic extracts of *S aromaticum* on *S.aureus*

Concentration (mg/ml)	Aqueous extract Diameters of the inhibition zone (mm)	Alcoholic extract Diameters of the inhibition zone (mm)
	St. Error ±Mean	St. Error ±Mean
0.00	0.00± 0.00	0.00± 0.00
12.5	0.15 ± 9.80	0.21 ± 13.00
25	0.55 ± 12.31	0.30 ± 16.33
50	0.31 ± 15.56	0.36 ± 18.33
100	0.23 ± 19.21	0.55 ± 20.31
200	0.70 ± 21.22	0.54 ± 24.04

($p<0.05$), Duncan test (Mean ± St. Error)

When comparing the result of the effect of aqueous and alcoholic extracts of *S. aromaticum* against *S. aureus* there were significant-differences ($P<0.05$) between them and the alcoholic extract was the best and this may be due to the ability of alcohol to extract the effective compound such as Glycosides, Alkaloids and Volatile dissolved oils. which have the ability to inhibit the growth of microorganisms via the ability to penetrate the cell wall or via its effect on vital parts of the cell wall such as Cytoplasm. Ribosome and DNA [33]. This is confirmed by the study of Al- Abdallal [34]. It has been found that the plant which belong to the Myrtaceae family have many active compounds with inhibitory effect against microorganisms [35]. The compounds work in different mechanisms, the phenolic compounds denaturate proteins in bacteria, flavonoids have the potential to penetrate though the bacterial cell wall and they are considered to be toxic oxidants compounds for microorganisms. It has been stated that the compounds of the Soaps, Glycoside and Tannins found the aqueous and alcohol extracts of Myrtle and Clove plants inhibit the action of enzymes responsible for metabolic reactions [36].

It is noted that the quantity and quality of the active ingredients of medicinal plants according to the conditions of plant development. In addition these compounds vary in their effect on microorganism subject to examination and conditions of the conducted expermints [37]. In our study. we tested the sensitivity of 35 isolates of *S. aureus* to 12 different antibiotics all isolates were sensitive 100% to Rifampin and Vancomycin. while the sensitivity to other antibiotics was 94.29% Oxacillin. In contrast, all isolates 100% were resistant to Penicillin G. (Table 4). The high resistance to Ampicillin and Penicillin G may be due to the fact that the bacterial isolates have Beta-lactamase that analysis of Penicillin or because they possess resistance gens on the chromosome or plasmid or as a result of mutation in Penicillin Binding Proteins [38].

Table 4: Antibiotics susceptibility of *S. aureus*

Antibiotic	Number of sensitive isolates and Percentage (%)		Number of resistance isolates and percentage (%)	
Amoxicillin/ Clavulanic acid	27	77.14%	8	22.86%
Ampicillin	0	0.00%	35	100%
Cefotaxime	15	42.86%	20	57.14%
Ciprofloxacin	29	82.86%	6	17.14%
Erthromycin	12	34.29%	23	65.71%
Gentamicin	19	54.29%	16	45.71%
Imipenem	32	91.43%	3	8.57%
Ofloxacin	24	68.57%	11	31.43%
Oxacillin	33	94.29%	2	5.71%
Penicillin G	0	0.00%	35	100%
Rifampin	35	100%	0	0.00%
Vancomycin	35	100%	0	0.00%

Recommendations

The aim of the present study was to discover new plant extracts that show inhibitory efficacy against different types of pathogenic microorganisms. The effects of extracts from *Myrtus communis L.* and *Syzygium aromaticum L.* against some pathogenic bacteria have been investigated. The current study suggests extraction and separation of the active ingredients of the *Myrtus communis L.* and *Syzygium aromaticum L.* and then testing their antimicrobial effect in laboratory animals

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