

Antibacterial Activity of *Punica granatum*, *Allium sativum* and *Piper nigrum* against Methicillin Resistant *Staphylococcus aureus*(MRSA) isolated from Wound Infections in Al-Hilla, Iraq

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Abstract : A total of 65 clinical samples were collected from patients suffering from wounds infections who admitted to Al-Hilla General Teaching Hospital. Among total 76bacterial isolates were identified,48(63.16%) isolates of *S. aureus* and all of them 48(100%) were methicillin resistant *S. aureus*(MRSA) depending on the results of antimicrobial susceptibility test of methicillin disc(5mg)and the cultivation on CHROM Agar MRSA Medium.

Antibacterial activities of ethanolic, methanolic and aqueous extract of *Punica granatum*, *Allium sativum* and *Piper nigrum* againsts MRSA. These three plants are mostly used in the prescriptions of folk medicine in Iraq.The antibacterial activity was measured by agar well diffusion Method and all extracts of *Punica granatum* showed high antibacterial activity with maximum inhibition zone (31-40)mm. Ethanolic and methanolic extracts of *Allium sativum* and *piper nigrum* have antibacterial activity, while their aquesous extracts have no antibacterial activity against MRSA.

Introduction

The rapid development of multi-drug resistance, limited antibacterial spectrum and adverse effects of available antimicrobial agents are becoming major causes of human mortality and morbidity¹.The incidence of *Staphylococcus aureus* infections and it's complications has increased abruptly in recent years because of the increased frequency of invasive procedures which has led to great number of immunocompromised patients and resistance of *S. aureus* strains to available antibiotics. This changing epidemiology of *S. aureus* infections, in combination with the inherent virulence of this pathogen, is commanding an urgent need for improved strategies and better antibiotics to prevent and treat *S. aureus*infections^{2,3}.

Introduction of methicillin into medical practice in the early 1960s quickly resulted in Methicillin-Resistant *S. aureus* (MRSA)⁴.Some MRSA are resistant to all but one or two antibiotics⁵. MRSA is any strain of *S.aureus* that has developed resistance to β -lactam antibiotics, which include the Penicillins(Methicillin, Dicloxacillin, Nafcillin, Oxacillin, etc.) and the Cephalosporins. Strains unable to resist these antibiotics are classified as Methicillin-Sensitive *S. aureus*(MSSA).The evolution of such resistance doesn't cause MRSA to be more intrinsically virulent than strains of MSSA but resistance does make MRSA infection more difficult to treat with standard types of antibiotics and thus more dangerous⁶.

Recently, the antimicrobial properties of certain indigenous plants were investigated and may yield useful results. This has consequently increased the attention and demand given to antimicrobials derived from the plants^{7,8}.Because oftheir “druglike” properties, i.e. their ability to be absorbed and metabolise, Natural

products, either as standardized plant extracts or as pure compounds, are expected to play an important role as one of the major sources of new drugs in the years to come⁹. The measurement of medicinal value of plants is based on the ability of production of some chemical substances also known as phytochemicals which produce a definite physiological action on humans or pathogen¹⁰.

In this research we using three different types of plant extracts: Methanoly, Ethanoly and Aqueous plant extract for different parts of three different medicinal plants, included: outer Peel of Pomegranate (*Punica granatum*), Garlic Bulbs(*Allium sativum*) and Seeds of Black Pepper(*Piper nigrum*).

The pomegranate(*Punica granatum*L.)was known for it's anti-cancerous, anti-inflammatory and anti-bacterial activity due to the different types of phytochemicals that have been identified from various parts of it'sfruit. Pomegranate pericarp(Peel, rind)has many important constituents; Hydrolyzable tannins(HTs) which are predominant polyphenols and has antioxidant activity¹¹. In addition, Phenolic punicalagins; gallic acid and other fatty acids; catechin, EGCG; quercetin, rutin and other flavonols; flavones, flavonones; anthocyanidins. The synergistic action of the pomegranate constituents appearsto be superior to that of single constituents¹².Garlic(*Allium sativum*) has traditional dietary and medicinal applications as an anti-infective agent, these applications were supported by In vitro evidences of the antibacterial, antifungal and antiviral activity of garlic extracts¹³.Black pepper (*Piper nigrum* L.) has piperamides which are the pungent bioactive alkaloids accumulate in the skin and seeds of the fruit. Among them piperine is the major chemical constituent responsible for the bitter taste of the black pepper and responsible of it's antimicrobial activity¹⁴.

Aim of this Study is investigation of the activities of three selected Iraqi medicinal plants with three different solvents, against MRSA clinical isolates, and investigation of the anti-Staphylococcus activity associated with botanicals historically used in the treatment of infections.

2.Materials and Methods

2.1. Isolation and identification

A total of 65 clinical samples were collected from patients suffering from wounds infections who admitted to Al-Hilla General Teaching Hospital. The samples were immediately inoculated on Blood Agar and Mannitol Salt Agar and incubated for overnight at 37°C under aerobic conditions. *S. aureus* isolates were diagnosed according to their characteristics and then compared with their characteristic being reported in referential references^{15,16}.CHROM Agar MRSA Mediumwas used for detection of Methicillin Resistant *S. aureus*(MRSA). This medium was prepared according to the instructions of the manufacturing company and cooled to about 50C°. 1ml of rehydrated CHRO Magar MRSA supplement was added to the prepared CHROMagar MRSA medium with slowly mixing. Rose to mauve colony indicates MRSA. While MSSA was inhibited and other bacteria was inhibited or colourless or blue colonies.

2.2. Preparation of Bacterial Suspension

About 18 hour colony on mannitol salt agar was suspended into sterile Brain Heart Infusion broth. It was standardized by gradually adding normal saline to compare their turbidity to McFarland standard.

2.3. Antimicrobial Susceptibility test

Susceptibility test for methicillin was performed by using the¹⁷ method on Muller-Hinton agar with the methicillin disc(5 mcg).An inoculum from a standard suspension of *S. aureus* of moderate turbidity equal to McFarland standard tube. A sterile swab was used to obtain an inoculum from the broth and streaked on a Muller-Hinton plate. The antibiotic discs were placed on the surface of the medium. Incubation overnight with 18-24 hrs at 37°C.in order to identify MRSA, Antibiotic inhibition zone surrounded methicillin disc was measured by using a ruler and compared to standard criteria in¹⁸.

2.4. Antibacterial activity of plant extracts

2.4.1. Preparation of Plant Extracts

Three plant samples were used in this study: Peel of Pomegranate, Garlic Bulbs and Seeds of Black Pepper were purchased from the local markets and air-dried at room temperature before grinding to powder with a mechanical grinder.

2.4.1.1. Aqueous Extraction(hot water):

10g of the weighed plant powder was soaked in 100ml of hot boiled water for 24hrs. The soaked extract was separated from the plant residue using filter paper and Buchner funnel, then it was centrifugated and the supernatant was filtered using Whatmann filter paper No.1. The filtrate was concentrated by using a Rotary Evaporator(Rotavapor R300) below 40°C for powdering the extract¹⁹.

2.4.1.2. Alcoholic Extraction

Alcoholic extracts were prepared for methanol and ethanol.

50g of the weighed plant powder was soaked in 250ml of 99.9% ethanol or methanol for 24hrs at room temperature. The soaked extract was separated from the plant residue using filter paper and Buchner funnel, then it was centrifugated and the supernatant was filtered using Whatmann filter paper No.1. The filtrate was concentrated by using a Rotary Evaporator (Rotavapor R300) below 40°C for powdering the extract^{19,20}.

2.4.1.3. Preparation of the Stock Solution and Dilutions

The powder of three extracts were stored in the refrigerator at 4 °C until required for use. 10g of each plant extract powder was dissolved in 50ml sterile distilled water in order to prepare the stock solution (200mg/ml). While, the ethanol extracts, were first dissolved in 1ml of 99.9% ethanol due to its inability to dissolve in water initially and oily nature. Then, further dilution in sterile distilled water to give ethanolic stock solution. Further filtration for stock solution by using millipore unit and whatmann filter paper No.1. stock solution was serially diluted with sterile distilled water to give three concentrations or dilutions(150, 100, 50)mg/ml¹⁹.

2.5. Antibacterial Activity of Plant Extracts on MRSA

The antibacterial activity of the crude extracts ON MRSA was determined in accordance with the agar-well diffusion method described by²¹. A sterile swab was used to obtain an inoculum from the Bacterial Suspension(which was prepared in 2.2) and streaked on a Muller-Hinton plate. Then, a hole with a diameter of (6)mm is punched aseptically with a sterile cork borer(No. 6). Approximately 50 µl of the crude extract at different concentrations (150,100,50)mg/ml were introduced into the wells. A negative control was prepared by putting 50 µl of sterile distilled water in one of the holes at the plates. One hour pre-diffusion time was allowed, after which the plates were incubated at 37°C for 18 h. The zones of inhibition were then measured in millimeter. The above method was carried out in duplicates and the mean of the duplicate results were taken^{22,23}.

3. Result and Discussion

3.1. Isolation and identification

A total of 76 bacterial isolates were identified from 65 clinical samples were collected from patients suffering from wound infections who admitted to Al-Hilla General Teaching Hospital. Laboratory diagnosis of bacterial isolates achieved according to the diagnostic characteristics and compared with those characteristics being reported in referential reference¹⁶. Among 76 bacterial isolates, 48 isolates of *S. aureus*(63.16%).

S. aureus isolates were cultivated on CHROM Agar MRSA Medium in order to detect Methicillin Resistant *S. aureus*(MRSA) presumptively.

All 48 isolates of *S. aureus* have rose to mauve colony on this medium.

Table 3.1: Inhibition Zone of Plant Extracts Against Methicillin Resistant Staphylococcus aureus (MRSA).

Medicinal plant	Type Of Extract	Inhibition zone (mm)				Negative Control
		200 mg/ml	150 mg/ml	100 mg/ml	50 mg/ml	
Peel of <i>Punicagranatum</i>	Ethanollic	40	35	33	31	0
	Methanolic	38	30	27	23	0
	Aqueous	25	21	20	17	0
Bulbsof <i>Allium sativum</i>	Ethanollic	36	34	33	28	0
	Methanolic	30	25	21	13	0
	Aqueous	0	0	0	0	0
Seeds of <i>Piper nigrum</i>	Ethanollic	27	23	20	18	0
	Methanolic	22	19	15	11	0
	Aqueous	0	0	0	0	0

3.2. Antimicrobial Susceptibility test

In order to detect the ability of *S. aureus* to resist methicillin, susceptibility test was performed by disc diffusion method with methicillin disc(5 mcg).The results of this experiment showed that, full resistance(100%) was observed against methicillin in all 48 isolates of *S. aureus*.

3.3. Antibacterial Activity of Plant Extracts on MRSA

Agar-well diffusion method was employed for determination of antibacterial activities of Aqueous, Ethanollic and Methanolic extracts of outer Peel of

Punica granatum, Garlic Bulbs of *Allium sativum* and Seeds of *Pipernigrum*. Quantitative evaluation of this activitywas carried out against MRSA by measuring of inhibition zone surrounded the wells containing the extract.

As showing in table 3-1., plant extracts have antibacterial activity against MRSA with clear differentaiton among extracts depending on concentration of extract, type of solvent,and type of plant.

As detailed in table 3.1, depend on the concentration of extract, the antibacterial effectiveness against methicillin resistant *S. aureus*of all extracts increases with increasing in concentration of extract, may be because of the increasing of concentration of active groups in the extract. It also supports the earlier investigations by previous published work^{24,25}.In this study, The results indicate that, ethanollic extracts have the highest inhibitory effect(largest inhibition zones) against MRSA as compared with methanolic and Aqueous extractst, table 3.1, may be because of the fact that, Ethanollic extraction of plants dissolves the organic compounds results in the liberation of the antimicrobial components²⁵.

This result was in agreement with²⁶ who mentioned that Ethanol extracts showed more activity against the bacteria than the water extracts. This may be due to the higher volatility of the ethanol which tends to extract more active compounds from the samples than water.

In the present study, the methanolic extract showed lower action than the ethanollic extract as antibacterial agents. This may be due to little diffusion properties of the extract in the agar or because fresh plants contain active substances which may be affected or attributed by the used solvent¹³.

As shown in table 3.1; Aqueous extracts have the lowest inhibitory effect, which is in agreement with²⁷ who reported that water may not be the most efficient reagent in extraction from pomegranate peels and seeds but more efficient extracting solvents like methanol,ethyl acetate, ethanol and butanol could be used.

As shown in table 3-1, Evidence is reviewed indicating a variation among studied plants in their inhibition activity of bacterial growth.

Punica granatum showed highest antimicrobial activity against MRSA with Inhibition zone ranged from (17-40)mm. All dilutions of this plant showed an inhibitory effect against MRSA. this result agree with²⁷ who emphasized that, pomegranate peel are good sources of phenolic compounds that have very potent antioxidant and antimicrobial activity. In addition, natural plant extracts contain a wide spectrum of phenolic phytochemicals. The most likely mechanism of antimicrobial activity by extracts containing phenolics has been postulated to be due to the disruption of the cell membrane.

As detailed in table 3.1: Maximum zone of inhibition was reported with Ethanolic extract of *Punica granatum* (31- 40)mm, followed by (23-38)mm for methanolic extract. While the minimum inhibition zone was reported with Aqueous extract (17-25)mm. The antibacterial effect of aqueous extracts in this study was comparatively less, but it is considered high, figure 3.1. this finding is in agreement with²⁸

Who reported that all extracts from pomegranate peels exhibited inhibitory activity against all test bacteria with the highest inhibition zones on ethanol extracts. On the other hand the antibacterial activity of ethanol extract of peels against *Aeromonas caviae* was not significantly different from other extracts of peels.

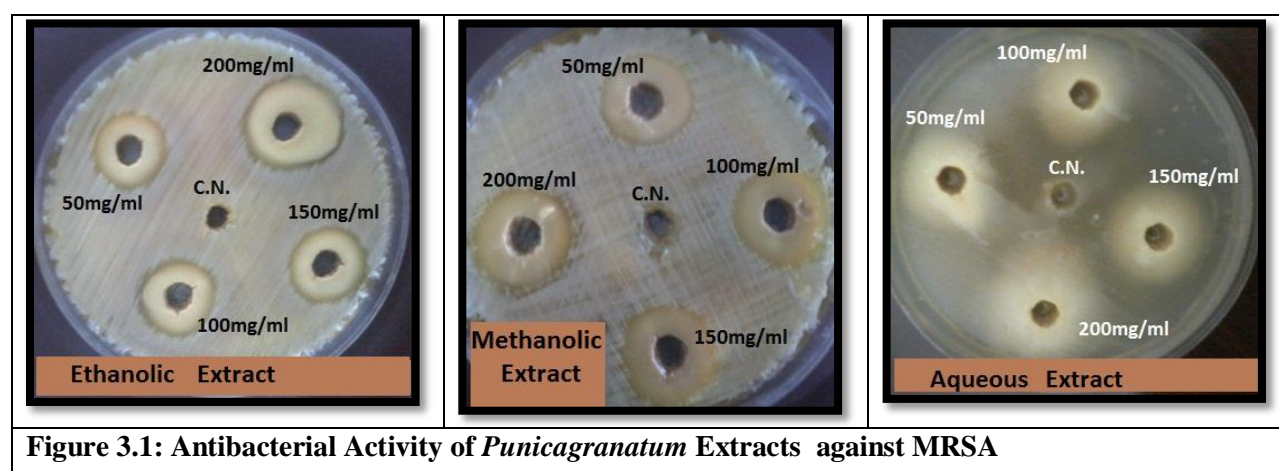


Figure 3.1: Antibacterial Activity of *Punica granatum* Extracts against MRSA

This finding is in disagreement with study by¹¹ which showed that the highest antibacterial activity against *S. aureus* was reported by methanolic extract of peel of pomegranate with inhibition zone (25)mm.

The potential therapeutic properties of *Punica granatum* are wide-ranging and include treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, diarrhea, and ulcers. In addition, it is used as an antiparasitic agent and provide the protection from ultraviolet radiation²⁹. On the other hand, several in vitro studies demonstrated the antibacterial activity of *Punica granatum* against several highly pathogenic and antibiotic-resistant bacteria. Based on researches by^{30,31}, the ellagitannin (punicalagin) is thought to be the primary constituent in peel of *Punica granatum* which is responsible for the observed antibacterial effects. The ellagitannins are a diverse class of hydrolyzable tannins³².

As detailed in table 3.1: *Allium sativum* extracts rank the second stage according to its antibacterial activity since its diameter of inhibition zone against MRSA with Inhibition zone ranged from (13-36)mm. Maximum zone of inhibition was reported with Ethanolic extract (28- 36)mm, followed by (13-30)mm for methanolic extract. While Aqueous extract has no inhibition zone (0.0 mg/ml), figure 3.2.

The garlic antibacterial activity on MRSA could be due to the action of biological active ingredient of allicin which exhibits its antimicrobial activity mainly by immediate and total inhibition of RNA synthesis, although DNA and protein syntheses are also partially inhibited, suggesting that RNA is the primary target of allicin action¹³. Allicin is an organosulfur compound which has been studied for its potential to treat various kinds of multiple drug resistance bacterial infections, as well as viral and fungal infections in vitro³³.

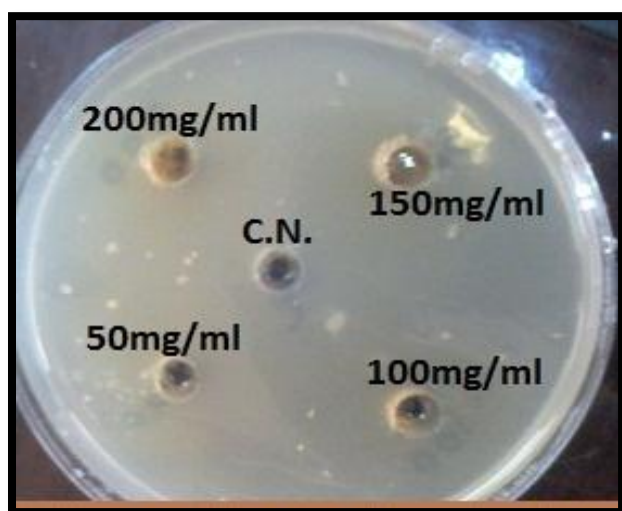


Figure 3.2: Antibacterial activity of aqueous extract of *Allium sativum* against MRSA

This findings are in contrast with³⁴ reported that crude extracts of *Allium sativum* did not exhibit any in vitro inhibition on the growth of tested organisms including *Staphylococcus*, and in disagreement with¹³ who found that the aqueous extract of *Allium sativum* had the highest inhibitory effects (19-48mm) against *S. aureus*.

In the present study, extracts of Seeds of *Piper nigrum* have the lowest antibacterial activity with smallest inhibition zones ranged from (11-27)mm. Its ethanolic extracts have maximum zones (18- 27)mm, followed by (11-22)mm for methanolic extracts. While Aqueous extract has no inhibition zone (0.0 mg/ml), table 3.1.

According to³⁵ The spicy taste of *Piper nigrum* due to the presence of piperamides which are the pungent bioactive alkaloids accumulate in the skin and seeds of the fruit. Among them piperine is the major chemical constituent.¹⁴ emphasized that the Piperine has antibacterial activity against all test bacteria with zone of inhibition ranged from (8-18)mm and the maximum zone of inhibition was against Gram positive bacteria *Staphylococcus aureus* (18mm).

Larhsiniet al.³⁶ and Sasidhran and Menon³⁷ describe the antimicrobial activity of volatile oils of *Piper nigrum* against bacteria and fungi. Volatile oil is one of the essential oils, and the studies have shown that essential oils may have the ability to prevent the transmission of some drug-resistant strains of pathogen, specifically *Staphylococcus*, *Streptococcus* and *Candida*³⁸.

Piper nigrum is also used in folk medicine as , stomachic, antiseptic, diuretic and for the treatment of cough, rheumatoid arthritis, peripheral neuropathy, melanoderma and leprosy due to the presence of volatile compounds, tannins, phenols and other unknown substances³⁹⁻⁴³.

Conclusion

From this study, It is concluded that all three studied plants have antibacterial activity against Methicillin Resistant *Staphylococcus aureus* (MRSA). *Punica granatum* showed highest antibacterial activity, followed by *Allium sativum*, and finally, *Piper nigrum*. It is a recommendation that natural products can use as therapeutic agents will probably not elicit resistance in bacteria and most research should continue to isolate and purify the active components and use in experimental animals.

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