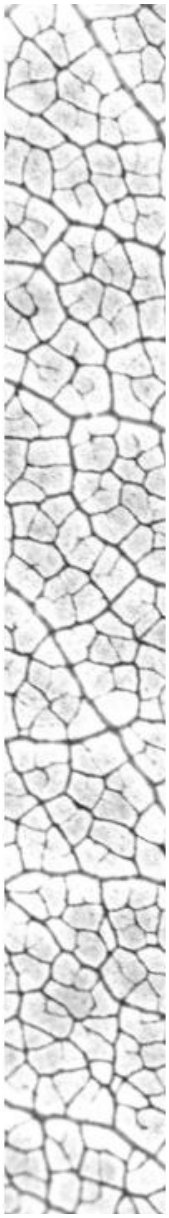

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Antibacterial screening for five local medicinal plants against nosocomial pathogens: *Klebsiella pneumoniae and Staphylococcus epidermidis*

Amal Talib Al-Sa'ady [1*]

[1] Asisst. Prof. PhD. Microbiology, Dept. Clinical Laboratory Sciences, College of Pharmacy, University of Babylon, Hillah, IRAQ
*Corresponding author: amal.atiyah@yahoo.com

Abstract

Introduction: The continuous increasing in the emergence of antibiotic-resistance in pathogenic bacteria has led to the development of new alternatives that are active against the pathogens. In Iraq, many plant components have been extensive attention as alternative medicine. **Materials and Methods:** *Punica granatum*, *Mentha pulegium, Allium cepa*, *Allium sativum* and *Piper nigrum* are five Iraqi medicinal plants were studied in this study. The plant samples were extracted in by three types of solvents, ethanol, methanol and hot boiled water. The plant extracts were examined as antibacterial agents against both *Staphylococcus epidermidis* and *Klebsiella pneumonia*e in four concentrations (50, 100, 150, 200 mg/ml) by using the agar-well diffusion method. **Results:** All assayed plant extracts have antibacterial activity in different concentrations and this activity was varied depending on some factors: the species of bacteria, the type of plant, the type of solvent and the concentration of extract. Depending on the species of bacteria, Gram negative bacteria has higher sensitivity than Gram positive against most studied plant extracts. The type of solvent has a clear effectiveness on the antibacterial activity, the ethanolic extract has the highest antibacterial activity of most studied plants, while the aqueous extract has the lowest activity. On the other hand, the increasing of extract concentration has been associated with increasing of the diameter of inhibition zone. Depending on the type of extracted plant, the maximum diameter of inhibition zone was yielded from *Punica granatum* extract regardless with the type of the solvent.

Keywords: antibacterial activity, Iraqi plants, *Klebsiella pneumoniae, Staphylococcus epidermidis*

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INTRODUCTION

The ever increasing drug resistance threats the health worldwide, and included most pathogenic bacteria and most common antibiotics. Plant-based therapies is an excellent alternative for antibiotics began to be offered in Iraq, the extracts of medicinal plants still used in traditional medicine. On the other hand, synergism may be done between Plant derived chemicals and antibiotics for enhancing the activity of antibiotics in order to make it low dosage and more potent.

Klebsiella pneumoniae is gram-negative rods, opportunistic and nosocomial pathogen which is responsible of many infections: sepsis, pneumonia, urinary tract infections, and hepatic abscess (Hennequin and Forestier 2007). Treatment of infections caused by multidrug-resistant *K. pneumoniae* became a significant challenge because it has high resistance to multiple broad-spectrum antibiotics (Al-Sa'ady and Naher 2018, Paterson et al. 2004). *Staphylococcus epidermidis* is a gram-positive, coagulase negative Staphylococci, an opportunistic pathogen has multi-drug resistance, produces the biofilm and associated with intravenous drug, catheter, prosthetic heart valves, shunts, prosthetic joints and causes many nosocomial infections: septicemia, endocarditis, UTI and meningitis (Le et al. 2014, Widerström et al. 2012). The increasing of the emergence of antibiotic-resistance in pathogenic bacteria has led to the development of new alternatives. In Iraq, many plants have been extensive attention as alternative medicines. In this study, four species of medicinal plants were studied: Iraqi pomegranate, Iraqi Butnig, Iraqi red Onion, Iraqi garlic and Black pepper.

Punica granatum L." Iraqi Roman" exhibits antivirus, antioxidant, anticancer, and anti-proliferative activities and successfully used against dysentery, diarrhea, Alzheimer's disease, male infertility and arthritis (Bassiri-Jahromi 2018). *Mentha pulegium* L "Iraqi Butnig" Has

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Table 1. Five Medicinal Iraqi Plant Samples used in the Present Study

No.	Local Name	Scientific Name	Part of Plant
1	Pomegranate (Iraqi Ruman)	*Punica granatum* L.	Peels
2	Iraqi Butnig (Dried Wild Mint)	*Mentha pulegium* L	Leaves
3	Iraqi red Onion	*Allium cepa* L.	Bulbs
4	Iraqi garlic	*Allium sativum*	Bulbs
5	Black pepper	*Piper nigrum* L	Fruits

many pharmacological effects against the disorders of gastrointestinal tract and it has anti-inflammatory, antiviral, antifungal, antimicrobial, and anti-carcinogenic activities (Ceyhan-Güvensen and Keskin 2016). *Allium cepa* L. "Iraqi red onion" contains phytochemical compounds such as allicin, which has anti-diabetic, antihypertensive, antibiotic and antithrombotic activities (Farag et al. 2017). *Allium sativum* "Iraqi garlic" has medicinal applications as an antimicrobial agent against fungi, viruses and bacteria such as, *Pseudomonas aeruginosa, methicillin-resistant Staphylococcus aureus, streptococcus sp., and mycobacterium Tuberculosis* (Reiter et al. 2017). *Piper nigrum* L "Black pepper" contains piperine, which is known for it' s remarkable pharmacological actions such as antioxidant, antitumor, antidiarrheal, anti-inflammatory, antifungal and antibacterial. On the other hand, it has exhibited significant antimicrobial activities against food spoilage bacteria and even antibiotic resistant bacteria (Aysa 2019, Salehi et al. 2019, Zou et al. 2015).

Aim of the Study

Evaluation of the antibacterial properties of *Punica granatum*, *Mentha pulegium, Allium cepa*, *Allium sativum* and *Piper nigrum* against both *Staphylococcus epidermidis* and *Klebsiella pneumoniae*, in vitro.

MATERIALS AND METHODS

Bacterial Isolation and Identification

During the period of October (2018) to March (2019), a total of 120 environmental samples were collected from Babylon Hospital for Pediatric and Gynecology/ Babylon / Iraq. these samples included swabs from hospital equipments, floor, walls, disinfectants, beds and Bathroom. Bacterial isolates were investigated for identification depending on their characteristics and API identification kits compared with referential references (Collee *et al.* 1996, MacFaddin 2000).

Collection of Plant Samples

Five medicinal Iraqi plant samples were collected from the local markets in Al- Hilla city/Iraq as detailed in **Table 1**. The plant samples were air-dried at room temperature before grinding to powder.

Preparation of Medicinal Plant Extracts

Alcoholic extraction

Methanol and ethanol were used to prepare alcoholic extracts by soaking 50g of dried plant powder in 250 ml of 99.9% alcohol at room temperature for 24 hrs. Filter paper and Buchner funnel were used for separating of the soaked extract from the plant residue. After centrifugation, the supernatant was filtered using What-man filter paper No.1 and concentrated by a Rotary Evaporator (Rota-vapor R300) below 40°C for powdering the extract (Fonkeng *et al.* 2015, Assefa et al 2016).

Aqueous extraction

100 ml of hot boiled water was used for soaking 10g of plant powder. Then, filtration by Buchner funnel and filter paper was done to separate the soaked extract from the plant residue. Next, it was centrifuged and the supernatant was filtered using What man filter paper No.1. Finally, for powdering the extract, the filtrate was concentrated by a Rotary Evaporator (Rota-vapor R300) under 40°C.

Preparation of Stock Solution and Dilutions

The powder of plant extract was stored in the refrigerator at 4ºC until required for use. Stock solution(200mg/ml) of aqueous and methanolic plant extract was prepared by dissolving of 10g of plant extract powder with 50ml sterile distilled water. While, the stock solution(200mg/ml) of ethanolic plant extract was prepared by dissolving of 10g of plant extract powder, initially, with 1ml of 99.9% ethanol and finally, with sterile distilled water. Further filtration for each stock solution by using Millipore unit and what man filter paper No.1. The serially dilutions were prepared for stock solution with sterile distilled water to give three dilutions (150, 100, 50) mg/ml.

Screening for Antibacterial Activity of Plant Extracts

Antibacterial activity of five types of plants was tested against *S. epidermidis* and *K. pneumoniae*. The bacterial suspension was prepared and standardized comparing to McFarland standard tube. Antibacterial activity of the plant extracts was evaluated by agar-well diffusion method. The surface of Muller-Hinton agar plate was streaked by swab of bacterial suspension. Then, a well with a diameter of (6) mm is punched aseptically by a sterilized cork borer (No. 6). Approximately 100 µl of each dilution (200, 150,100,50) mg/ml was introduced into the wells. A negative control was done by introducing 100 µl of sterile distilled water into the fifth well. One-hour pre-diffusion time was allowed at room temperature(16°C). Incubation at 37°C for 18h.The inhibition zone was measured in millimeter. It was carried out in duplicates and the mean of the duplicate results were taken.

RESULTS AND DISCUSSION

Last decades, many natural substances have been determined as a source of the effective antibacterial agents and the studies have focused on the plants as potential antimicrobial agents. Plants are rich in a broad spectrum of secondary metabolites such as, alkaloids,

Table 2. Diameter of Inhibition zones of five plant extracts at 200 mg/ml against *S. epidermidis* and *K. pneumoniae*

Type of Extract	The Plant	*Staphylococcus epidermidis*	*Klebsiella Pneumoniae*
Ethanolic	*Punica granatum*	35*	45
	Mentha pulegium	22	38
	Allium cepa	18	30
	Allium sativum	30	19
	Piper nigrum	23	20
Methanolic	*Punica granatum*	25	40
	Mentha pulegium	18	33
	Allium cepa	23	35
	Allium sativum	7	25
	Piper nigrum	7	25
Aqueous	*Punica granatum*	15	25
	Mentha pulegium	9	27
	Allium cepa	0	23
	Allium sativum	0	0
	Piper nigrum	0	0
The range of diameter of inhibition zone		7-35 mm	19-45 mm

*The data in the table = the mean of the duplicate results were taken.

flavonoids, tannins and terpenoids which have been demonstrated to have antibacterial activities for controlling pathogenic bacteria (Al Saimary et al. 2002).

In the present study, all assayed plant extracts have antibacterial activity in different concentrations against both *K. pneumonia*e and *S. epidermidis*, **Table 2** and **Fig. 5**. These findings are in agreement with many studies suggested that, these medicinal plants can be used as alternative therapeutic agents for controlling of many common disease conditions (Ceyhan-Güvensen and Keskin 2016, Fuchs *et al*. 2018, Salehi *et al*. 2019, Sharifi-Rad et al., 2016, Vučić *et al*. 2019).

This antibacterial activity may be attributed for the presence of broad-spectrum of antibiotic compounds or simply general metabolic toxins. Previous studies concluded that the plant extracts has anti-biofilm activity along with antibacterial activity (Lee *et al.* 2013). Usually, the comparison among results in different studies is not fair because there is no standardized approaches for the test conditions, such as size of the inoculum, technique of inoculum preparation, conditions of the bacterial growth and no standardized of the diameters for the inhibition zones or the endpoints, in addition to, the varied conditions of plant cultivation and extraction (Balouiri *et al.* 2016).

The results in the present study confirmed that, the antibacterial activity for the studied plant extracts was varied depending on some factors, such as the species of bacteria, the type of plant, the type of solvent, the concentration of extract. This is in agreement with Kyung (2012) who described that, the solvent type, the concentration of extract, and extraction method have a chief role in determining the antibacterial activity of plant extracts.

Depending on the species of bacteria, Gram-ve bacteria has higher sensitivity than Gram+ve bacteria against most studied plant extracts regardless with the type of solvent. As detailed in **Table 2**, the diameters of inhibition zones in crude extract for *K. pneumoniae* ranged (19-45)mm, while the diameters of inhibition zones for *S. epidermidis* ranged(7-35)mm. This variation may be due to the difference in the cell wall and cell membrane compositions between Gram+ve and Gram-negative bacteria. This finding is in agreement with Ceyhan-Güvensen and Keskin (2016) while is in disagreement with Yildirim *et al* (2013) where found that the gram+ve bacteria commonly seem to be more susceptible to the inhibitory effects of the plant extracts than the gram-ve bacteria. While Elisha *et al.* (2017) concluded that the plant extracts which have high antibacterial activity against Gram-negative bacteria do not necessarily have high activity against other Gram-positive bacteria.

In the present study, the type of solvent has a clear effectiveness on the antibacterial activity of the studied plant extracts, as illustrated in **Fig. 1** and **Fig. 2** and **Fig. 5f**. The ethanolic extract has the highest antibacterial activity of most studied plants, followed by the methanolic extract, while the aqueous extract has the lowest activity, **Fig. 5**. This finding was in comparable to numerous studies have investigated that the solvent has great influence on the composition of extract by the different phytochemicals were found in different solvents and the ethanolic extracts gave better inhibitory action against bacteria (Sajjad *et al.* 2015). It is noteworthy that, the ethanolic extracts for *Punica granatum, Mentha*

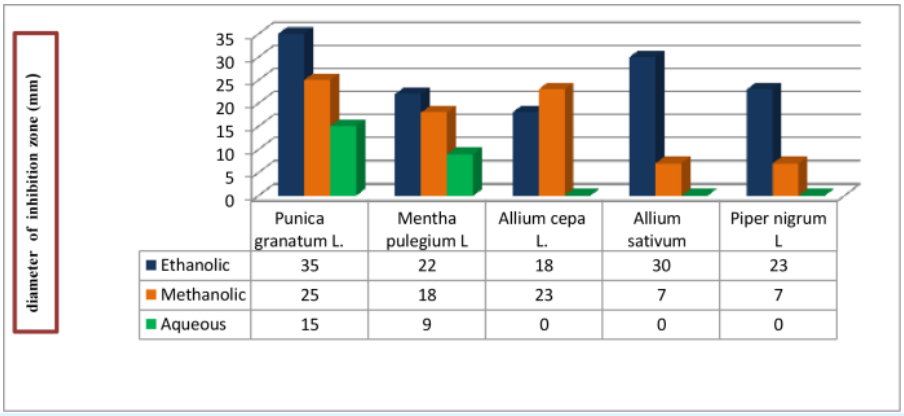
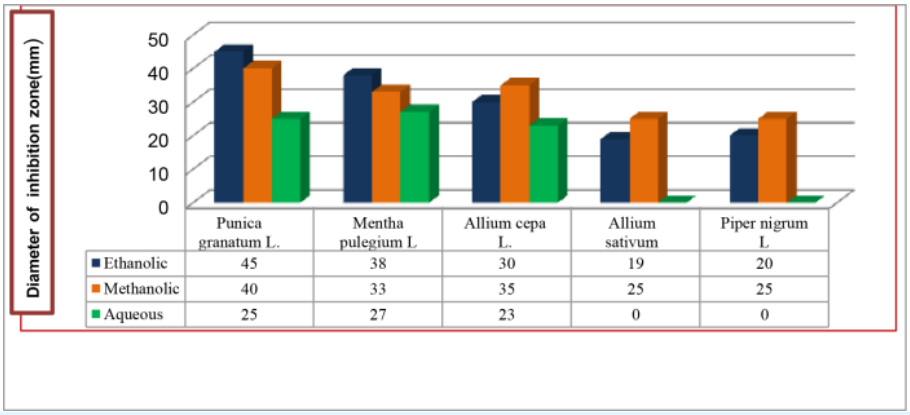
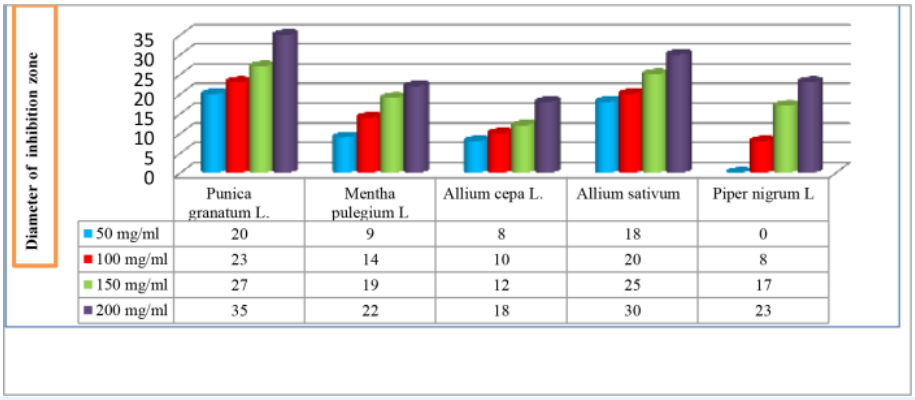


Fig. 1. Antibacterial Activity of five plant extracts against *Staphylococcus epidermidis* at stock concentration(crude) 200 mg/ml.



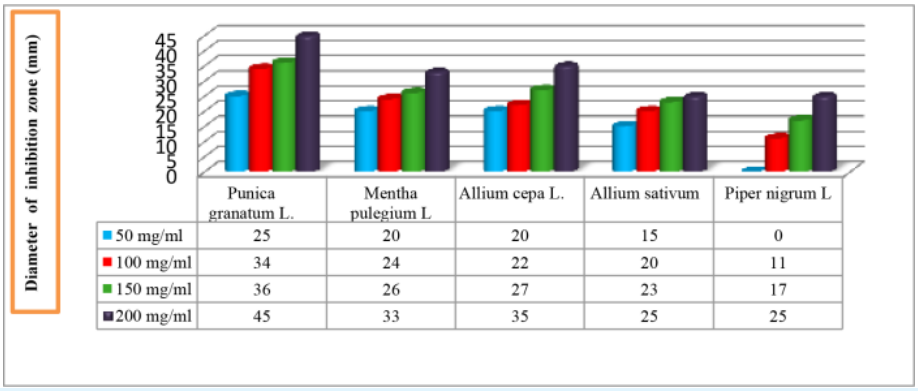
	Punica granatum L.	Mentha pulegium L	Allium cepa L.	Allium sativum	Piper nigrum L
Ethanolic	45	38	30	19	20
Methanolic	40	33	35	25	25
Aqueous	25	27	23	0	0

Fig. 2. Antibacterial Activity of five plant extracts against *klebsiella pneumoniae* at stock concentration (crude) 200 mg/ml



	Punica granatum L.	Mentha pulegium L	Allium cepa L.	Allium sativum	Piper nigrum L
50 mg/ml	20	9	8	18	0
100 mg/ml	23	14	10	20	8
150 mg/ml	27	19	12	25	17
200 mg/ml	35	22	18	30	23

Fig. 3. The antibacterial activity of ethanolic extracts against *Staphylococcus epidermidis* was rising with the increasing of concentration



	Punica granatum L.	Mentha pulegium L	Allium cepa L.	Allium sativum	Piper nigrum L
50 mg/ml	25	20	20	15	0
100 mg/ml	34	24	22	20	11
150 mg/ml	36	26	27	23	17
200 mg/ml	45	33	35	25	25

Fig. 4. The antibacterial activity of methanolic extracts against *klebsiella pneumoniae* was rising with the increasing of concentration

pulegium, Allium sativum and *Piper nigrum* has higher bioactivity than the methanolic extracts for the same plants. While the methanolic extract for *Allium cepa* has higher antibacterial activity than its ethanolic extract, **Table 2**.

As shown in **Figs. 3**-**5**. The increasing of extract concentration has been associated with increasing of the diameter of inhibition zone and subsequently the antibacterial activity has increased. Ascribable to the increasing of concentration of phytochemicals and active groups along with the increasing of the extract concentration. In the present study, the crude extract (200 mg/ml) has the highest activity, for example, in **Fig. 3** ethanolic extract of *Punica granatum* has inhibition zones against *S. epidermidis* with diameters 20, 23, 27, 35 mm in 50, 100, 150, 200 mg/ml, respectivly. On the other hand, in **Fig. 4** methanolic extract of *Punica granatum* has inhibition zones against *K. pneumoniae* with diameters 25, 34, 36, 45 mm in 50, 100, 150, 200 mg/ml, respectivly. These findings were in agreement with many recent studies (Al Sa'ady and Al-Mawla 2019, Debalke *et al.* 2018, Shah *et al.* 2017).

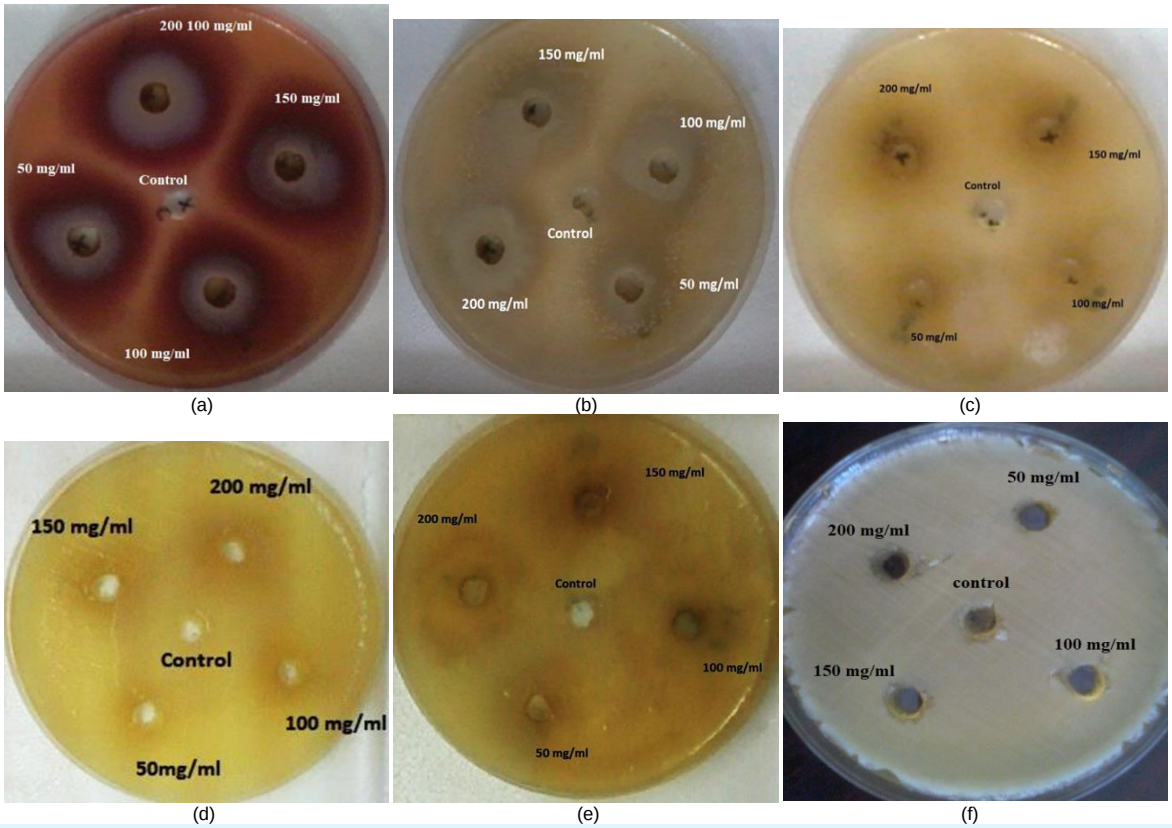


Fig. 5. The antibacterial activity for plant extracts by agar well diffusion method on the Agar Mueller Hinton**.**
a. Antibacterial Activity of ethanolic extract of *Punica granatum* against *K. pneumonia*
b. Antibacterial activity of methanolic extract of *Punica granatum* against *K. pneumonia*
c. Antibacterial Activity of aqueous extract of *Punica granatum* against *K. pneumonia*
d. Antibacterial Activity of ethanolic extract of *Mentha pulegium* against *K. pneumonia*
e. Antibacterial Activity of methanolic extract of *Allium cepa* against *K. pneumonia*
f. No antibacterial Activity of aqueous extract of *Piper nigrum* against *K. pneumonia* and *S. epidermidis*

Depending on the type of extracted plant, the maximum diameter of inhibition zone was yielded from *Punica granatum* extracts regardless with the type of the solvent, **Table 2**, **Fig. 5a**-**c**. The ethanolic and methanolic and aqueous extracts for *Punica granatum* revealed antibacterial activity higher than other studied plant extracts with diameter of inhibition zones 45, 40, 25 mm respectively against *K. pneumonia*, **Fig. 2**, and 35, 25, 15 mm respectively against *S. epidermidis* **Fig. 1** this is in agreement with other studies who mentioned that *P. granatum* was the most effective extract with bacteriostatic and bactericidal activities. Approximately every part of the pomegranate has wide ranges of phytochemical properties have demonstrated antibacterial activities. Ellagic acid and hydrolysable tannins, such as punicalagin, have the most activities (Howell and D'Souza 2013, Kadhum et al. 2017, Mostafa et al. 2018).

CONCLUSION

It is concluded that, the five studied medicinal plants possess clear antibacterial activity against both Gram negative and Gram positive pathogenic bacteria which supports the role of these plants in the traditional usage as alternative treatment. This antibacterial activity was influenced by several factors, such as the species of bacteria, the type of plant, the type of solvent and the concentration of extract.

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