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The Antimicrobial Effect of Alcoholic Extract of *Peganumharmala* L Seeds on Clinically Isolated Gram Negative and Gram Positive Bacteria

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Abstract

The aim of the present study was assess the antimicrobial effect of *Peganumharmala* L seeds extracts by ethanol (80%) on gram negative and gram positive bacteria and four concentrations (25, 50, 75 and 100) mg/ml were prepared. Four clinical isolates of bacteria were used; two were positive and two were negative bacteria; that include: *Bacillus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The results showed that all concentration that have been used had antimicrobial effect against gram negative and gram positive bacteria and the best concentration that have the best antimicrobial effect was 100 mg/ml and the effect of alcoholic extraction was greater on gram positive bacteria than gram negative bacteria, also the antimicrobial effect of two antibiotics were tested on these four clinical isolates these antibiotics are imipeneme and gentamycin. The effect of imipeneme was greater than the effect of gentamycin also the effect of imipeneme on gram positive bacteria was greater than on gram negative bacteria. The synergistic effect of alcoholic extraction of *Peganumharmala*L seeds and antibiotics was studied and the result show that the antimicrobial effect of antibiotics (imipeneme and gentamycin) was increased when these antibiotics discs were saturated with 100mg/ml of seeds extracts. The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the alcoholic extracts of *Peganumharmala* L seeds on these four clinical isolates were tested and the result show that the MIC was: for *Bacillus* : 3.12 mg/ml , *Staphylococcus aureus*:1.56 mg/ml, *Pseudomonas aeruginosa*: 6.25 mg/ml and *Escherichia coli*:3.12 mg/ml) and the MBC was :(for *Bacillus* : 3.12 mg/ml , *Staphylococcus aureus*:3.12 mg/ml, *Pseudomonas aeruginosa*: 12.5 mg/ml and *Escherichia coli*: 6.25 mg/ml.

Keywords: *Peganumharmala* extract, Imipeneme, gentamycin, MIC, MBC.

التأثير الضد مايكروبي للمستخلص الكحولي لبذور الحرمل *Peganumharmala* على البكتريا الموجبة والسالبة لصبغة غرام المعزولة سريريا

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الخلاصة:

تهدف هده الدراسة الى تقييم التأثير المضاد للبكتريا للمستخلص الكحولي لبذور الحرمل *Peganumharmala* ضد البكتريا الموجبة والسالبة لصبغة غرام. استخدم الايثانول 80% في الاستخلاص وتم تحضير أربعة تراكيز من المستخلص هي:25,50,75و100ملغم/مل استخدمت ضد البكتريا الموجبة والسالبة لصبغة غرام وهي :*BacillusStaphylococcus aureus, Pseudomonas aeuroginosa,* و*Eschirichia coli*. اوضحت النتائج إن جميع التراكيز كان لها تأثيرا مضادا للبكتريا الموجبة والسالبة

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لصبغة غرام، وان أفضل تثبيط للبكتريا عند تركيز 100 ملغم/مل. وان تأثير المستخلص الكحولي على البكتريا الموجبة لصبغة غرام أكثر من البكتريا السالبة لصبغة غرام. أوضحت النتائج ايضا ان المضادات الحيوية المستخدمة في البحث وهي:imipeneme, gentamycin لها تأثيرا مضادا للبكتريا المعزولة المستخدمة في البحث، وان تاثير المضاد imipeneme كان اكثر من تأثير المضاد gentamycin على البكتريا كما وجد ان imipeneme له تأثير على البكتريا الموجبة اكثر من السالبة لصبغة غرام.درست ايضا التأثيرات التآزرية للمستخلص الكحولي لبذور الحرمل مع المضادات الحيوية(imipeneme, gentamycin) وأوضحت النتائج ان التأثير المضاد للبكتريا من قبل المضادات الحيوية يزداد عندما تتشبع أقراص المضاد الحيوي بتركيز 100 ملغم/مل من المستخلص الكحولي للحرمل.اختبر تأثير التركيز المثبط الأدنى والتركيز البكتيري الأدنى للمستخلص الكحولي على البكتريا المعزولة وأوضحت النتائج إن التركيز المثبط الأدنى لبكتريا *Bacillus* كان 3,12 ملغم/مل وبكتريا *S. aureus* كان 1,56 وبكتريا *P. aeroginosa* كان 6,25 ملغم/مل وبكتريا *E. coli* كان 3,12 ملغم/مل، وان التركيز البكتيري الادنى كان لبكتريا *Bacillus* 3,12 ملغم/مل وبكتريا *S. aureus* كان 3,12 ملغم/مل وبكتريا *P. aeuroginosa* كان 12,5 ملغم / مل بينما بكتريا *E. coli* كان 6,25 ملغم/مل.

Introduction

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects [1]. Plant extracts have been used since the dawn of civilization by mankind [2]. Natural products are believed to be an important source of new chemical substance with potential therapeutic applicability [3]. *Peganumharmala* L. (Zygophyllaceae), distributed mainly in the Mediterranean region, also found in Central Asia, North Africa and also cultivated in America and Australia, it is commonly called as Esfand and Suryin Rue [2].*Peganumharmala*is a perennial, bushy and wild-growing flowering plant with short creeping root which may grow to 30-100 cm high and native from the eastern Iranian region west to India. A red dye, from the seeds is often used in western Asia to dye carpets and wool. The stems, roots and seeds are used to make inks, stains and tattoos [4]. It is rich in alkaloids of type β-carboline and contains up to 2 - 7% total alkaloids [1]. Several studies have shown various biological activities and pharmacological characteristics of its seeds such as hypothermia, hallucinogen factor, antidepressant, inhibitor of the enzyme monoamine oxydase (MAO), antibacterial, antifungal and anti-virus. It is effective for the treatment of dermatosis disease, its leaves are used as an antinociceptive .However, it causes abortion in rats [2].

P. harmala extracts are considered important for drug development, because they are reported to have numerous pharmacological activities in the Middle East, especially in Iran and Egypt, for a long time *P. harmala* has been used in traditional medicines for the relief of pain and as an antiseptic agent [2]. *P. harmala*also have antibacterial, antifungal, antiviral, antioxidant, antidiabetic, antitumor, antileishmanial, insecticidal and cytotoxic activities and hepatoprotective and antinociceptiveeffects [5]. The aims of the study are: use the alcoholic extract of *Peganumharmala* seeds as a antimicrobial agents against gram negative and gram positive bacteria and study the synergistic effect between this extract and antibiotic against bacterial isolates.

Materials and Methods

Bacterial isolates

Four clinical bacterial isolates were used, two were positive bacteria and two were negative bacteria as shown Table -1.

Table 1-The bacterial isolates, specimens, clinical sources and the places of isolation

Bacterial isolates	Specimen	Clinical sources	Place
A- Gram positive bacteria			
Bacillus spp.	soil	-	Central laboratory of biology department/college of science. Baghdad, Iraq
Staphylococcus aureus	Cotton swab	Nose, from patient suffering from respiratory tract infection	Al- Yarmuk Teaching Hospital. Baghdad. Iraq.
B- Gram negative bacteria			
Pseudomonas aeuroginosa	Cotton swab	Wound, from patient suffering from wound infection	Al- Yarmuk Teaching Hospital. Baghdad. Iraq.
Escherichia coli	Urine	From patient suffering from urinary tract infection	Al- Yarmuk Teaching Hospital. Baghdad. Iraq.

All the isolates were diagnosis according to their morphological characters and biochemical tests. MacConkey agar (Himedia/India) was used in order to primarly identification of *P. aeruginosa* and *E. coli* isolates, also oxidase test was used in diagnosis of *P. aeruginosa* that was prepared according to Collee *et al.*[6] by dissolving 1g of N, N, N, N-tetramethyl-p-phenylene-diamine dihydrochloride (BHD/ England) in 100 ml of D.W., stored in dark bottle and used immediately. Blood agar (Himedia /India) was used in identification of *Staph. aureus* isolate, for the detection of hemolytic activity and the kind of hemolysis. Mannitol salt agar (Himedia /India) was also used for identification and isolation of *Staph. aureus*. Nutrient agar(Himedia /India) was used in identification of *Bacillus.* The diagnosis ofthe clinical isolates was confirmed by Api 20 E system for *P. aeruginosa* and *E. coli* and Api staph system for *Staph. aureus* (bio-Mieruk/ France).

Antibiotics sensitivity test

Two antibiotics were used imipenem 10mcg and gentamicin 30mcg (Bioanalyse). Antibiotic sensitivity test of clinical isolates was done by Baures and Kirbys [7] disc diffusion method. Organisms were grown in Mueller Hinton broth MHB (Himedia /India) for 18 hrs. at 37ºC and inoculation on Mueller Hinton agar MHA (Himedia/India) plates by sterile swabs after dilution to (1×10^8 cell/ml) and then antibiotics discs were placed on media and pressed gently followed by overnight incubation at 37ºC. The results were comparing with CLIS [8] data.

Alcoholic extract preparation from *Peganumharmala* seeds:

The dry seeds of *Peganumharmala* were purified washed and dried under fresh air, then ground in electrical grinder to get fine powder of the seeds. Each 10 g of the ground seeds were used with 200 ml of 80% ethanol in Soxhletapparatuse for 8 hrs, then the extracts were dried in rotary evaporator, and the resulted powder kept in tightly closed glass container in refrigerator until used to prepare different concentrations 25, 50, 75 and 100 mg/ml.

Antibacterial assay procedure

Agar diffusion technique was applied to study the antimicrobial effect of the alcoholic seeds extract of *P. harmala* on clinical isolates on MHA. At first, a total of 0.1 ml of bacterial suspension that were cultured over night at 37 °C in the MHB and used as inoculums then the turbidimetry of the suspension was adjusted to the McFarland 0.5 turbidity standard (10^8cfu/ml) was poured on each plate containing MHA [9]. The lawn culture was prepared by sterile cotton swab and allowed to remain in contact for 1 min. A well method was employed by making holes using cork borer in the MHA (5 mm in diameter and 4 mm in depth). Each well was filled with 40µl of selected agents and allowed to stand for 1 hrs. at room temperature to diffuse the plant extracts into medium before incubation at 37˚C for 24 hrs. Inhibition zones were across the diameter of each well. Complete resistance of bacterial isolates to the tested agent was indicated when there were no zones of inhibition [10].The Petri dishes were incubated at 37 °C for 24 hrs and the inhibition zone around each well was measured in mm. This experiment was carried out in duplicate. Wells with 80 % methanol was also included to test if they had an inhibitory effect on the test bacteria.

MIC and MBC determination

The MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) of methanol extracts from the seeds of *P. harmala* were determined against the four clinical isolates. MIC was determined by the macro broth dilution assay method [11]. In the tube dilution assay, standard bacterial suspension (0.1 ml) and 1 ml of different concentrations of extract (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50,100 and 200 mg/ml) were added to tubes containing 1 ml MHB. These tubes were incubated at 37 °C for 24 hrs. The first tube in the above series without sign of visible growth was considered as the MIC. MBC was determined by culturing one standard loop of the tubes with no apparent growth on MHA and subsequent incubation at 37 °C for 24 hrs. The least concentration that inhibited colony formation on agar was considered as MBC for the extract.

Study of the synergistic effect between alcoholic seeds extract and antibiotics

To determine the synergistic effect of the most effective concentration of alcoholic seeds extract of *P. harmala* with synthetic antibiotics (imipenem and gentamicin) 100 mg/ml of the extract was added to the discs containing these antibiotics and their effect was evaluated by disc diffusion method on the clinical isolates [12].

Statistical Analysis

The Statistical Analysis System- SAS [13] program was used to effect of Bacterial isolate in study parameters. Least significant difference-LSD test was used to significant compare between means.

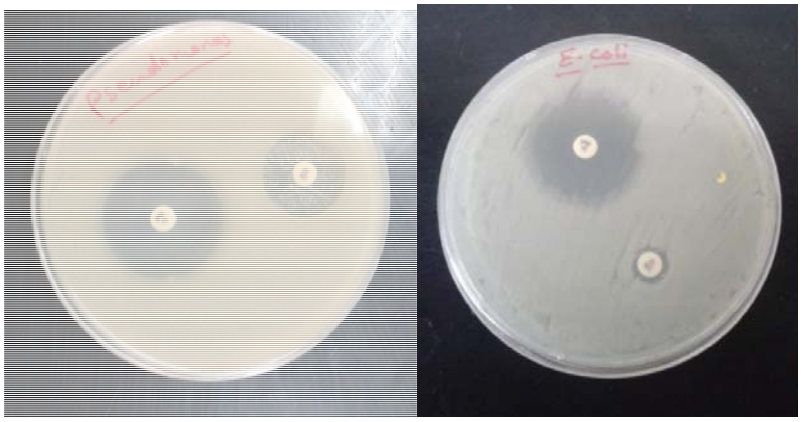
Results and Discussion:

Four clinical isolates were used in this study, two of them were gram negative bacteria: *Pseudomonas aeruginosa* that was isolated from patients suffering wound infection and *E. coli* that was isolated from patient suffering from urinary tract infection in Al-Yarmuk teaching Hospital (Baghdad, Iraq).The two others were gram positive bacteria: *Staphylococcus aureus* that was isolated from patient suffering from respiratory tract infection in Al-Yarmuk teaching Hospital (Baghdad, Iraq) and *Bacillus* that was obtained from Central laboratory of biology department/ Collage of sciences/ University of Baghdad. The *Pseudomonas aeruginosa* and *E. coli* isolateswere diagnosed primarily by growing on MacConkey agar.*E. coli* colonies appeared dry, small and pink colonies because it ferment lactose while the *P. aeruginosa* colonies appeared smooth and pale because it does not ferment lactose. The *P. aeruginosa* colonies were tested for their ability to produced oxidase enzyme by using oxidase test, the isolate gave positive result by giving deep-purple color on filter paper after added oxidase reagents. Then the diagnosis for both isolates were confirmed by Api 20 E system. *Staphylococcus aureus* isolate was diagnosed primarily on Blood agar, their colonies gave large, round and β-hemolyic and on Mannitol salt agar the grow and gave yellow colonies because of their ability to produce golden pigment and ferment sugars then the diagnosis was confirmed by Api staph system.

The results of the sensitivity test of the two antibiotics (imipenem and gentamicin) show that two antibiotics have antimicrobial effect on gram positive bacteria and the imipenem has greater antimicrobial effect than the gentamicin as shown in figure 1but on gram negative bacteria only the imipenem has antimicrobial effect as shown in figure -2.

A **B**

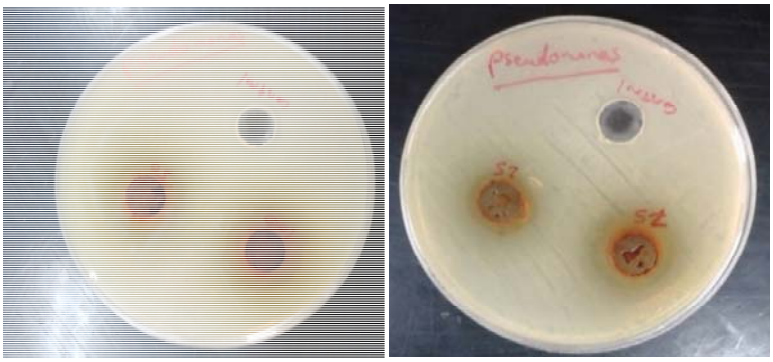
Figure 1-The effect of antibiotics (imipenem and gentamicin) on gram positive bacteria **A**: The effect of antibiotics (imipenem and gentamicin) on ***Staphylococcus aureus***.**B:** The effect of antibiotics (imipenem and gentamicin) on ***Bacillus.***



A **B**

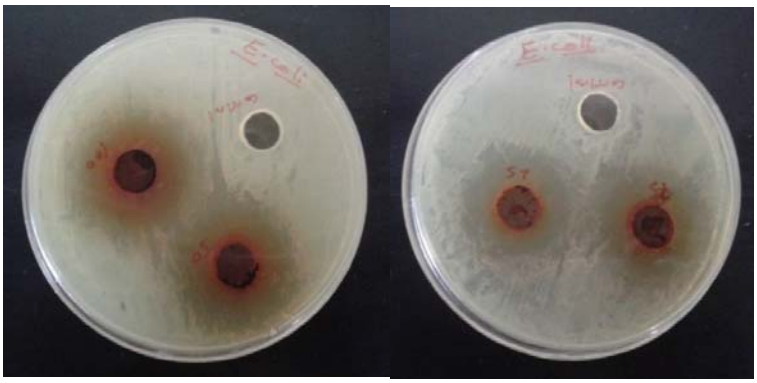
Figure 2-The effect of antibiotics (imipenem and gentamicin) on gram negative bacteria.**A**: The effect of antibiotics (imipenem and gentamicin) on ***Pseudomonas aeuroginosa***. **B:** The effect of antibiotics (imipenem and gentamicin) on ***E. coli.***

Also the results show that the four concentration (25, 50, 75 and 100) mg/ml of alcoholic extraction of *P. harmala* L seeds have antimicrobial effect on gram positive and gram negative bacteria in compare with the control as shown in figure -3, -4,- 5 and 6and the best concentration was 100mg/ml. The effect of alcoholic extraction on gram positive bacteria was better than on gram negative bacteria.



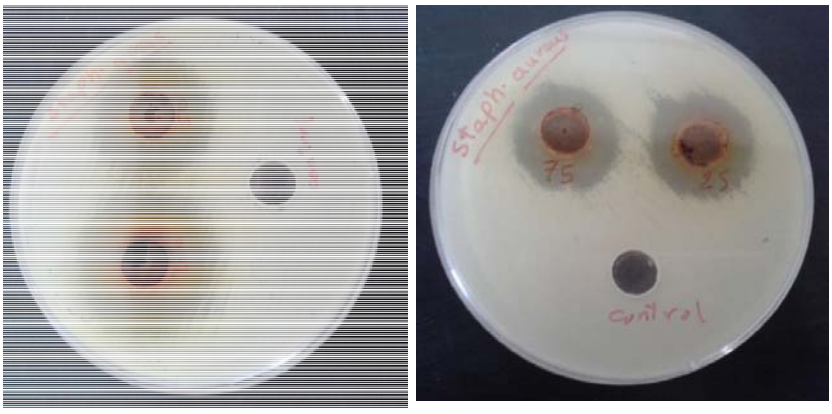
A **B**

Figure 3-The effect of the four concentrations of alcoholic extraction of ***Peganumharmala*** L seeds on ***Pseudomonas aeruginosa***. **A:** The effect of 50 and 100mg/ml concentrations of alcoholic extraction of *Peganumharmala* L seeds on ***Pseudomonas aeruginosa***. **B:** The effect of 25 and 75mg/ml concentrations of alcoholic extraction of *Peganumharmala* L seeds on ***Pseudomonas aeruginosa***.



A **B**

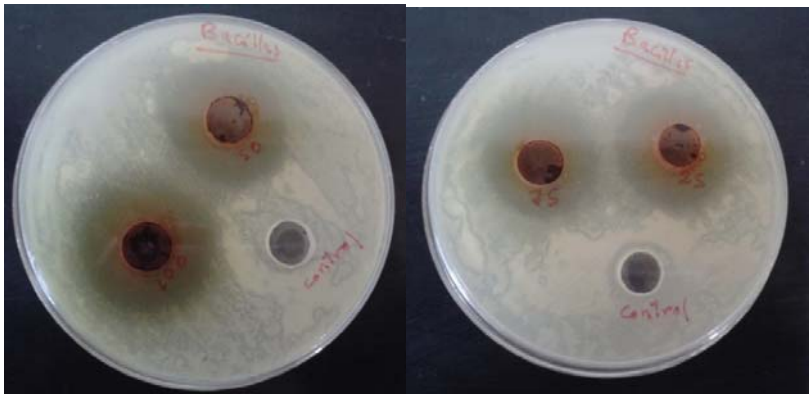
Figure 4-The effect of the four concentrations of alcoholic extraction of ***Peganumharmala*** L seeds on ***E. coli***. **A:** The effect of 50 and 100mg/ml concentrations of alcoholic extraction of *Peganumharmala* L seeds *on* ***E. coli.*** **B:** The effect of 25 and 75mg/ml concentrations of alcoholic extraction of *Peganumharmala* L seeds on ***E. coli***.



A **B**

Figure 5-The effect of the four concentrations of alcoholic extraction of ***Peganumharmala*** L seeds on ***Staphylococcus aureus***. **A:** The effect of 50 and 100mg/ml concentrations of alcoholic extraction of

Peganumharmala L seeds on ***Staphylococcus aureus***. **B:** The effect of 25 and 75mg/ml concentrations of alcoholic extraction of *Peganumharmala* L seeds on ***Staphylococcus aureus***.



A **B**

Figure 6:-The effect of the four concentrations of alcoholic extraction of ***Peganumharmala*** L seeds on ***Bacillus***. **A:** The effect of 50 and 100mg/ml concentrations of alcoholic extraction of *Peganumharmala* L seeds on ***Bacillus***. **B:** The effect of 25 and 75mg/ml concentrations of alcoholic extraction of *Peganumharmala* L seeds on ***Bacillus***.

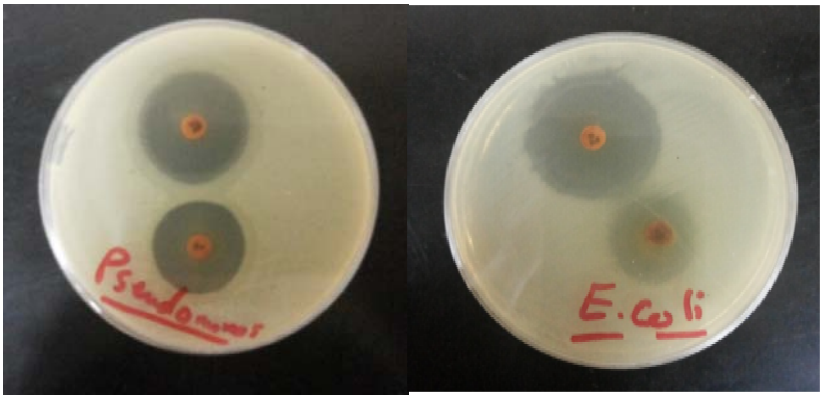
The MIC and MBC of alcoholic extraction of *P. harmala* L seeds on gram positive and gram negative bacteria were studied and values are listed in table -2.

Table 2-The MIC and MBC of Alcoholic extraction of *Peganumharmala* L seeds for gram positive and gram negative bacteria

Bacterial isolate	**MIC (mg/ml)**	**MBC (mg/ml)**
Bacillus	3.12 ± 0.07	3.12 ± 0.02
Staphylococcus aureus	1.56 ± 0.02	3.12 ± 0.04
E. coli	3.12 ± 0.07	6.25 ± 0.41
Pseudomonas aeruginosa	6.25 ± 0.32	12.5 ± 0.71
LSD value	1.092 *	1.177 *
* (P<0.05).		

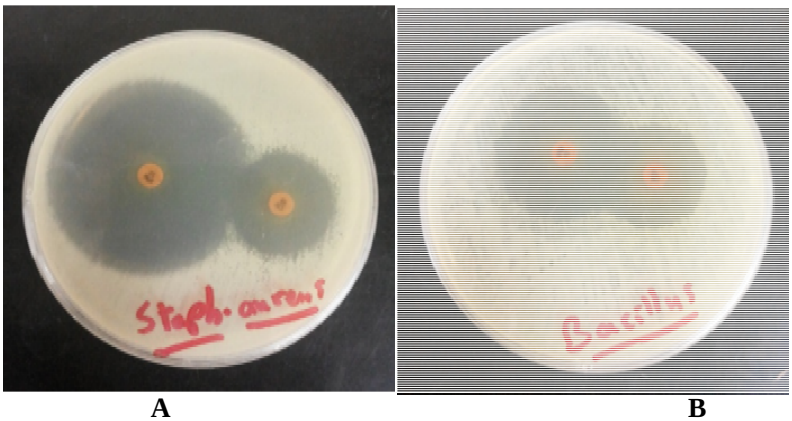
The statistical analysis of study results showed that there was no significant differences between MIC for *Bacillus* and *E. coli*, but significant differences between MIC for other bacteria (P<0.05), also the results showed that there was no significant differences between MBC for *Bacillus* and *Staphylococcus aureus*, but significant differences between other bacteria used in this study (P<0.05) as showed in table -2.

Also the results show that the saturation of antibiotics discs with 100 mg/ml of alcoholic extract of *P. harmala*L seeds increased the antimicrobial effect of these antibiotics on gram negative and gram positive bacteria as shown in figure -7 and -8. It is obvious that the gentamicin that have no antimicrobial effect on gram negative bacteria has antimicrobial effect when it saturated with alcoholic extraction also the effect of imipenem was highly enhanced.



A **B**

Figure 7-The synergistic effect of antibiotics (imipenem and gentamicin) on gram negative bacteria. **A:** The synergistic effect of antibiotics (imipenem and gentamicin) on ***Pseudomonas aeruginosa***. **B:** The synergistic effect of antibiotics (imipenem and gentamicin) on ***E. coli.***



A **B**

Figure 8-The synergistic effect of antibiotics (imipenem and gentamicin) and alcoholic extract of *Peganumharmala* seeds on gram positive bacteria.**A:** The synergistic effect of antibiotics (imipenem and gentamicin)and alcoholic extract of *Peganumharmala* seeds on ***Staphylococcus aureus***.**B:** The synergistic effect of antibiotics (imipenem and gentamicin) and alcoholic extract of *Peganumharmala* seeds on ***Bacillus.***

P. harmala seeds have been considered from ancient time to date as a plant with drug usages regarding to some alkaloids compounds such as harmalin and harmalol. The compounds extracted from this plant have shown different medical characteristics [14]. *Peganumharmala* has "antibacterial activity," including antibacterial activity against drug-resistant bacteria[15].The results obtained showed that the plant *P. harmala* almost prevents the growth of all microorganisms tested, but to varying degrees of effectiveness. Concentration of 100mg/ml of crude extract of seeds inhibits the growth of all bacterial strains studied. Recording the highest microbial activity with the crude extract of the seeds against the Gram positive bacterial strains (*Bacillus*, *S. aureus*), compared to Gram negative strains and this is due the strong resistance of Gram negative bacterial strains to antibiotics [16] which showed that alcoholic extract of the plant *P. harmala*is very effective with Gram positive bacteria. These results are agreed with the results that recorded by Benbott*et al*.[17]. According to the results of the present study, alcoholic extracts of Harmel seeds possess antimicrobial activity, and the most sensitive strain is *S. aureus* with a diameter of 25 mm of the inhibitory zone for the ethanol extract. Whereas the most resistant strain is *P. aerogenosa* with a diameter of 12 mm of the inhibition zone for the ethanol extract. This later shows that *P. aeroginosa* is well known to be very resistant to many antimicrobial agents and antibiotics in general which is probably due to the capacity of bacteria to form a bio-film or a polysaccharide barrier. This barrier is a complex organization composed of different strata connected from the internal to the external membrane where the bacteria are found in a specific physiological state to their situation. Therefore, all the bacterial population is not

simultaneously and identically exposed to the product. It is established that the treatment of such bacteria require considerable concentrations of antimicrobial agents [18].

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