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 to 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.

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

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Specimen</th>
<th>Clinical sources</th>
<th>Place</th>
</tr>
</thead>
<tbody>
<tr>
<td>A- Gram positive bacteria</td>
<td>Bacillus spp.</td>
<td>soil</td>
<td>Central laboratory of biology department/college of science. Baghdad, Iraq</td>
</tr>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
<td>Cotton swab, Nose, from patient suffering from respiratory tract infection</td>
<td>Al-Yarmuk Teaching Hospital. Baghdad, Iraq</td>
</tr>
<tr>
<td>B- Gram negative bacteria</td>
<td>Pseudomonas aeruginosa</td>
<td>Cotton swab, Wound, from patient suffering from wound infection</td>
<td>Al-Yarmuk Teaching Hospital. Baghdad, Iraq</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>Urine, From patient suffering from urinary tract infection</td>
<td>Al-Yarmuk Teaching Hospital. Baghdad, Iraq</td>
</tr>
</tbody>
</table>
All the isolates were diagnosed according to their morphological characters and biochemical tests. MacConkey agar (Himedia/India) was used in order to primarily 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-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 of the 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 *Peganum harmala* seeds:**

The dry seeds of *Peganum harmala* 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 Soxhlet apparatus 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 (10^8 cfu/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* isolates were 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 produce 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-hemolytic 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 1 but on gram negative bacteria only the imipenem has antimicrobial effect as shown in figure 2.

![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*.](image)

![Figure 2: The effect of antibiotics (imipenem and gentamicin) on gram negative bacteria. A: The effect of antibiotics (imipenem and gentamicin) on *Pseudomonas aeruginosa*. B: The effect of antibiotics (imipenem and gentamicin) on *E. coli*.](image)
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 6 and the best concentration was 100mg/ml. The effect of alcoholic extraction on gram positive bacteria was better than on gram negative bacteria.

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.

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.

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 and B](image)

**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

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
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<tbody>
<tr>
<td><em>Bacillus</em></td>
<td>3.12 ± 0.07</td>
<td>3.12 ± 0.02</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1.56 ± 0.02</td>
<td>3.12 ± 0.04</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>3.12 ± 0.07</td>
<td>6.25 ± 0.41</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>6.25 ± 0.32</td>
<td>12.5 ± 0.71</td>
</tr>
<tr>
<td>LSD value</td>
<td>1.092 *</td>
<td>1.177 *</td>
</tr>
</tbody>
</table>

* (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.
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*.

Figure 8-The synergistic effect of antibiotics (imipenem and gentamicin) and alcoholic extract of *Peganum harmala* seeds on gram positive bacteria. A: The synergistic effect of antibiotics (imipenem and gentamicin) and alcoholic extract of *Peganum harmala* seeds on *Staphylococcus aureus*. B: The synergistic effect of antibiotics (imipenem and gentamicin) and alcoholic extract of *Peganum harmala* 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]. *Peganum harmala* 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 alcholic extract of the plant *P. harmala is* very effective with Gram positive bacteria. These results are agreed with the results that recorded by Benbottet al.[17]. According to the results of the present study, alcholic 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. aerogenosa* 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].

References