Investigation of the Antibacterial Effect of Native *Peganum harmala*, *Mentha pulegium* and *Alcea rosea* Hydro-alcoholic Extracts on Antibiotic Resistant *Streptococcus pneumoniae* and *Klebsiella pneumonia* Isolated from Baku, Azerbaijan

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Background: Pneumonia and respiratory tract infections, is associated with high mortality and complications in humans. Current antibiotics are used to treat this infectious disease, but may lead to many problems such as unwanted side effects and resistance to antibiotics. This study investigated the antibacterial activity of the hydro alcoholic extracts of the native medicinal plants *Peganum harmala*, *Mentha pulegium* and *Alcea rosea*, in Baku, as a natural alternative to antibiotics, on antibiotic-resistant *Streptococcus pneumoniae* and *Klebsiella pneumonia*, the main bacteria that cause pneumonia.

Materials and Methods: Antibacterial activity of the hydro alcoholic extracts of medicinal part of these plants was evaluated by the disk diffusion susceptibility test method and the broth dilution test method on bacteria.

Results: The rate of MIC of *P. harmala*, *M. pulegium* and *A. rosea* extracts of *S. pneumoniae* were 80, 110 and 375μgL⁻¹ and for *K. pneumoniae* were 150, 230 and 680μgL⁻¹ respectively, and the rate of MBC were 120, 165 and 550μgL⁻¹ for *S. pneumoniae* and 210, 315 and 800μgL⁻¹ for *K. pneumoniae* respectively. The maximum amount of inhibition zone diameter in 500μgL⁻¹ concentration of *P. harmala*, *M. pulegium* and *A. rosea* extracts for *S. pneumoniae* were 21.2mm, 17.2mm, 6.9mm and for *K. pneumoniae* were 10.1mm, 8.1mm, 3.2mm, respectively.

Conclusion: This work showed that substances in the hydro-alcoholic extracts of medicinal plants prevented the growth of bacteria. So these plants having effective ingredients can be used as an affordable and available source for medicinal purposes.

Keywords: Pneumonia, *Peganum harmala*, *Mentha pulegium*, *Alcea rosea*, Baku, Azerbaijan

1. Background

Respiratory diseases include pathological conditions affecting the organs and tissues that are part of gas exchange process in higher organisms, and involves both upper and lower regions consisting of the nose, pharynx, and other structures such as the middle ear and sinuses. The lower portion of the system comprises of the respiratory tubes and alveoli of the lungs. The excessive moisture, and rich supply of nutrients in this area, makes it ideal for infections to grow. *Streptococcus pneumoniae*, a diplococcus, gram-positive, alpha-hemolytic with polysaccharide capsule, and *Klebsiella pneumoniae*, gram-negative, non-motile, encapsulated, lactose fermenting, rod shaped bacteria, are the main facultative bacteria which cause pneumonia in humans. Respiratory tract infections caused by these pathogenic bacteria are associated with high mortality and complications (1-3).

Although antibiotics are used to treat common infectious diseases, but these treatments are associated with many problems such as unwanted side effects and development of resistance. Plants can be considered as a substitute for chemical drugs, since they have fewer side effects. Today, extensive researches have been carried out in traditional medicine, including herbal medicine in different fields of medical sciences (4).

Investigations on the essential oil and extract of *Mentha pulegium* (English name= Pennyroyal), belonging to family Lamiaceae, shows significant impacts in preventing the growth of several species of spoilage and pathogenic agents due to its anti-bacterial, anti-inflammatory and anti-spasmodic activity (5-7). *Alcea rosea* (English name= Russian Hollyhock), belonging to family Malvaceae, is herbaceous and perennial plant that reaches a height of about 2 meters. The leaves are wide, serrated, like the heart. It has large yellow flowers (8). *Peganum harmala* (English name= harmala), from Zygophyllaceae family, has been one of the popular herb in traditional medicine and has been effective due to its anti-bacterial, anti-fungal, anti-parasitic, sleeping, sweating, aborting the fetus, anti-cancer, immune system stimulating and mono amino oxidase enzyme inhibitors (9).

2. Objectives

This study aimed to determine the antibacterial in vitro effects of native *Mentha pulegium*, *Alcea rosea* and *Peganum harmala* hydro-alcoholic extracts as natural alternatives to antibiotics, on *K. pneumoniae* and *S. pneumoniae*, isolated from Baku, Azerbaijan.

3. Materials and Methods

3.1. Preparation of plant extracts

Hydro-alcoholic extraction was performed by maceration method. First of all, 50g of the leaves of pennyroyal, the seeds of harmala and the flower of Russian Hollyhock were prepared and dried in oven and then powdered. The powders of plants were poured into the flasks, separately. For each sample 1500mL solvent [50% Water and 50% ethanol (96%)] was added, so as to cover the powder completely. Then, the flasks were covered with aluminum foil. Flasks were shaken for 48 h at 90rpm. Then, the homogeneous solutions were filtered by filter paper and finally rotary evaporator was used to separate solvent from the extract. The purified extracts were stored in the refrigerator for further experiment (4).
3.2. Preparation of microorganisms
Antibiotic-resistant bacteria were prepared from cultures isolated in several hospitals in Baku. The lyophilized standard strains S. pneumoniae ATCC49619 and K. pneumoniae ATCC13883 were prepared from ATCC reference center. In order to preparation of bacteria from lyophilized samples, first samples were cultured in a nutrient broth overnight at 30-35°C. After the turbidity samples were isolated and purified on blood agar and MacConkey agar, respectively (10, 11).

3.3. Antibacterial susceptibility testing
Antibacterial activity of extracts was evaluated by the agar-disk diffusion method. Overnight bacterial suspensions were first adjusted to 0.5 McFarland turbidity standards (approximate concentration: 1.5×10^8 CFU mL^-1). The bacterial suspensions were transferred to Muller Hinton agar plates using a sterile swab (one swab), aseptically. Sterile blank disks (diameter 6mm) were impregnated by 20μl of the extracts in dilutions 62.5, 125, 250 and 500μg mL^-1. Then impregnated disks were completely dried in laboratory temperature overnight. The disks impregnated with the solvents were considered as controls. These disks were placed on Mueller Hinton agar medium containing bacteria by pence in regular intervals. Plates containing bacterial cultures and extracts were incubated at 37°C for 18-24 hours. Antibacterial activity was evaluated by the Clinical and Laboratory Standards Institute (CLSI) guidelines. Each experiment was carried out in triplicate and the mean difference between rates of MIC and MBC with level of significance (P-value <0.05).

3.4. Determination of inhibitory activity of extracts
The minimum inhibitory concentration (MIC) values were determined for the bacterial strains based on a macrodilution method which were sensitive to the extracts in the disk diffusion assay. overnight cultures were used to make The inoculums of the bacterial strains; suspensions were adjusted to 0.5 McFarland standard turbidity. The mixtures of bacteria (one loop-full) and dilutions of the extracts were incubated at 37°C for 24 hours in 1mL Muller Hinton broth. After this period, the concentration of the first tube without turbidity was considered as MIC. In the next stage, the contents (one swab) of the non-growth tubes were cultured in Muller Hinton agar plates. After incubation at 37 °C for 24 hours the first non-growth plate was considered as minimum bactericidal concentration (MBC). Controls were as follows: i) Medium and extract without bacteria: non-growth, ii) medium and distilled water with bacteria: growth, iii) medium and chlorhexidine (positive control) with bacteria: non-growth (3, 10, 11, 13).

Moreover, binary combination as well as the sum of all three plants was prepared with an equal volume of dilution 500μg mL^-1 in order to observe the synergy of plants and tested with mentioned similar experiments.

4. Results
The results of antibacterial activity of native M. pulegium, A. rosea and P. harmala hydro-alcoholic extracts on antibiotic-resistant K. pneumonia and S. pneumoniae by the disk diffusion agar susceptibility test and broth macrodilution test methods is presented below (P-value <0.05). The results of the inhibition zone diameter obtained from effect of different concentrations of hydro-alcoholic extracts of medicinal plants on antibiotic-resistant bacteria are shown in Table1. The standard diameter of inhibition zone of antibiotics ampicillin, ciprofloxacin, nitrofurantoin and vancomycin are 26≤, 21≤, 17≤ and 12≤mm for sensitive S. pneumoniae ATCC49619 and that of ciprofloxacin, tetracycline, amoxicillin and nitrofurantoin are 21≤, 19≤, 18≤ and 17≤ mm for sensitive K. pneumonia ATCC13883. The amount of all controls was θ± 0,0mm.

The results of broth macrodilution test method is presented in Table 2 which shows the difference between rates of MIC and MBC with level of significance (P-value <0.05) native medicinal plant extracts in Baku on S. pneumoniae and K. pneumoniae. Table 3 shows the results of the different combined effect of plant extracts on bacteria.

### Table 1. The diameter of non-growth zone of different concentrations of plants hydro-alcoholic extracts on antibiotic-resistant bacteria.

<table>
<thead>
<tr>
<th>Concentration (μg mL^-1)</th>
<th>S. pneumoniae</th>
<th>K. pneumoniae</th>
<th>S. pneumoniae</th>
<th>K. pneumoniae</th>
<th>S. pneumoniae</th>
<th>K. pneumoniae</th>
<th>A. rosea</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5</td>
<td>7.1±0.7</td>
<td>3.4±0.4</td>
<td>5.9±0.9</td>
<td>2.7±0.6</td>
<td>2.1±0.5</td>
<td>1.2±0.6</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>11±1.8</td>
<td>6±1.2</td>
<td>10±2.2</td>
<td>5±1.7</td>
<td>2.8±0.6</td>
<td>2.1±0.8</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>16±2.2</td>
<td>8±1.8</td>
<td>13±2.6</td>
<td>6±1.6</td>
<td>3.7±0.2</td>
<td>2.9±0.2</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>27±1.9</td>
<td>10±1.2</td>
<td>17±2.8</td>
<td>8±1.8</td>
<td>6.9±0.8</td>
<td>3.2±0.4</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. The amount of (MIC) and (MBC) of medicinal plants extracts on bacterial species (μg mL^-1).

<table>
<thead>
<tr>
<th>P. harmala</th>
<th>M. pulegium</th>
<th>A. rosea</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pneumoniae</td>
<td>K. pneumoniae</td>
<td>S. pneumoniae</td>
</tr>
<tr>
<td>MIC</td>
<td>MIC</td>
<td>MIC</td>
</tr>
<tr>
<td>MBC</td>
<td>MBC</td>
<td>MBC</td>
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<tr>
<td>80</td>
<td>120</td>
<td>150</td>
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</tbody>
</table>

### Table 3. The mean of concentration 500 μg mL^-1 of combined with an equal volume of medicinal plants hydro-alcoholic extracts on the non-growth zone (mm), MIC and MBC (μg mL^-1).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>P. harmala and M. pulegium</th>
<th>P. harmala and A. rosea</th>
<th>M. pulegium and A. rosea</th>
<th>All three plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pneumoniae</td>
<td>Non-growth zone (mm)</td>
<td>22±1.1</td>
<td>18±1.6</td>
<td>14±1.7</td>
</tr>
<tr>
<td>MIC (μg mL^-1)</td>
<td>95</td>
<td>100</td>
<td>135</td>
<td>95</td>
</tr>
<tr>
<td>MBC (μg mL^-1)</td>
<td>135</td>
<td>150</td>
<td>185</td>
<td>135</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>Non-growth zone</td>
<td>9.2±1.4</td>
<td>7.9±1.1</td>
<td>6.1±1.5</td>
</tr>
<tr>
<td>MIC</td>
<td>160</td>
<td>170</td>
<td>280</td>
<td>165</td>
</tr>
<tr>
<td>MBC</td>
<td>230</td>
<td>250</td>
<td>390</td>
<td>255</td>
</tr>
</tbody>
</table>
5. Discussion
The use of herbs at various eras and nations has played important role in the treatment of different diseases (2, 4, 5, 7, 9, 11). The native medicinal plants extract is provided as a source of antimicrobial compounds. Hence, in this work the antibacterial effects of native M. pulegium, A. rosea and P. harmala, in Baku; hydro-alcoholic extracts were investigated on bacteria that cause pneumonia. In antibacterial susceptibility test the inhibitory diameters raised with increasing of extracts concentration, significantly; the controls in these experiments had not any antimicrobial effect. The maximum effects involved in 500μg mL⁻¹ that were 27.2mm (P. harmala), 13.2mm (M. pulegium) and 6.9mm (A. rosea) in S. pneumoniae and 10.1mm (P. harmala), 8.1mm (M. pulegium) and 3.2mm (A. rosea) in K. pneumoniae. The rates of MIC and MBC of extracts were 80μg mL⁻¹ and 120μg mL⁻¹ (P. harmala), 110μg mL⁻¹ and 165μg mL⁻¹ (M. pulegium) and 375μg mL⁻¹ and 550μg mL⁻¹ (A. rosea) in S. pneumoniae, 150μg mL⁻¹ and 210μg mL⁻¹ (P. harmala), 230μg mL⁻¹ and 315μg mL⁻¹ (M. pulegium) and 680μg mL⁻¹ and 800μg mL⁻¹ (A. rosea) in K. pneumoniae, respectively. The appropriate concentration of P. harmala extract against S. pneumoniae could be really effective in comparison with antibiotics; although other extracts, contrary to local belief, had minor effects. Observations showed that the combination of these extracts did not create special synergy effect. Sensitivity of S. pneumoniae against all 3 plant extracts was the most prominent compared with K. pneumonia. The P. harmala had the greatest impact; and in the next step were M. pulegium and A. rosea, respectively. Essential oils obtained by hydrodistillation from the leaves of Mentha pulegium L. and Mentha rotundifolia (L.) Huds. from Uruguay have been analyzed by GC-FID and GC-MS. Oxygen-containing monoterpenes had the main group of constituents in both oils. Pulegone, isomenthol and menthone comprised the major components in the oil of M. pulegium, whereas piperitenone oxide and (Z)-sabinene hydrate included the major ones in M. rotundifolia. Enantiomerically pure (+)-menthone, (+)-isomenthone, (+)-isomenthol, (-)-menthol and (+)-pulegone had been detected by multidimensional gas chromatography in the case of M. pulegium oil (12). Harmala as a plant with medicinal properties of having active compounds such as harmaline, harmine and harmalol alkaloids have traditionally been considered. Most studies on the therapeutic properties of the harmala seed have been associated with parasitic infections. Based on findings alcoholic extract of seeds of Peganum showed the growth measurable inhibitory and fatal activity on yeast Candida measurable (13). The research has suggested that the ethanol extract of P. harmala, as an antioxidant, reduces free radicals as a result of the use of silver nanoparticles (9). This work was also concluded that the appropriate concentration of P. harmala extract against S. pneumoniae could be really effective than antibiotics; although the other extracts, contrary to local belief, had minor effects. Observations showed that the composition of these extracts did not create a special synergy effect.

6. Conclusion
Each year in the mountainous areas in Baku, the large amount of variety of herbs grew self-propelled, particularly our plants in this work, and later destroyed without any use. With regard to the effective ingredients of these plants in our study and other researches in the growth inhibition of bacteria, simply, could be exploited as an affordable and available source of biopharmaceuticals.

Conflict of Interests
Authors declare they have no conflict of interests.

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Authors’ Contributions
All authors contributed extensively to the work presented in this paper.

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References