Infect Epidemiol Med. 2016 Spring; Volume 2, Issue 2: 12-14 DOI: 10.7508/iem.2016.02.004
Published online 2016 Spring **Original Article**

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

Mehdi Ghasemi*, Yemen Atakishiyeva

Institute of Microbiology, Azerbaijan National Academy of Sciences, Baku, Azerbaijan

*Corresponding author: Mehdi Ghasemi, Institute of Microbiology, Azerbaijan National Academy of Sciences, Baku, Azerbaijan. Tel: +989353680735, E-mail: mehdi_aidin@yahoo.com

Submitted: December 7, 2013; **Revised:** February 11, 2014; **Accepted:** March 8, 2014

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 pneumoniae*, 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 375µgµL^{-1} and for *K. pneumoniae* were 150, 230 and 680µgµL^{-1} respectively, and the rate of MBC were 120, 165 and 550µgµL^{-1} for *S. pneumoniae* and 210, 315 and 800µgµL^{-1} for*K. pneumonia* respectively; The maximum amount of inhibition zone diameter in500µgµL^{-1} concentration of *P. harmala*, *M. pulegium* and *A. rosea* extracts for *S. pneumoniae* were 21.2mm, 17.2mm, 6.9mmand for *K. pneumonia* were10.1mm, 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 with 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 encompasses pathological conditions affecting the organs and tissues that make gas exchange possible in higher organisms, and includes 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 consists of the respiratory tubes and alveoli of the lungs. Infection occurs here because of the excessive moisture, and rich supply of nutrients. *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 causes 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 Holly-hock), 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 CFUmL^{-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 500µgµL^{-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 37ºC 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 diameter of the inhibition zone was recorded (10-13).

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. The inoculums of the bacterial strains were prepared from overnight cultures; 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 500µgµL^{-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 0± 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.

Concentration (µgµL^{-1})	Diameter of non-growth zone (mm) in medicinal plants					
	P. harmala		*M. pulegium*		*A. rosea*	
	S. pneumoniae	*K. pneumonia*	*S. pneumoniae*	*K. pneumonia*	*S. pneumoniae*	*K. pneumonia*
62.5	7.1±0.7	3.1±0.4	5.9±0.9	2.7±0.6	2.1±0.5	1.2±0.6
125	11.3±1.8	6.8±1.2	10.9±2.2	5.1±1.7	2.8±.0.6	2.1±.0.8
250	16.3±2.2	8.9±1.8	13.2±2.6	6.4±1.6	3.7±0.2	2.9±0.2
500	27.2±1.9	10.1±1.2	17.2±2.8	8.1±1.8	6.9±0.8	3.2±0.4

Table 2. The amount of (MIC) and (MBC) of medicinal plants extracts on bacterial species (µgµL^{-1}).

P. harmala				*M. pulegium*				*A. rosea*			
S. pneumoniae		*K. pneumoniae*		*S. pneumoniae*		*K. pneumoniae*		*S. pneumoniae*		*K. pneumoniae*	
MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
80	120	150	210	110	165	230	315	375	550	680	800

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

Bacteria		*P. harmala* and *M. pulegium*	*P. harmala* and *A. rosea*	*M. pulegium* and *A. rosea*	All three plants
S. pneumoniae	Non-growth zone (mm)	22.2±1.1	18.2±1.6	14.8±1.7	21.2±1.1
	MIC (µgµL^{-1})	95	100	135	95
	MBC (µgµL^{-1})	135	150	185	135
K. pneumoniae	Non-growth zone	9.2±1.4	7.9±1.1	6.1±1.5	9.7±1.5
	MIC	160	170	280	165
	MBC	230	250	390	255

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 500µgµl^{-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. pneumonia*. The rates of MIC and MBC of extracts were 80µgµL^{-1} and 120µgµL^{-1} (*P. harmala*), 110µgµL^{-1} and 165µgµL^{-1} (*M. pulegium*) and 375µgµL^{-1} and 550µgµL^{-1} (*A. rosea*) in *S. pneumoniae*, 150µgµL^{-1} and 210µgµL^{-1} (*P. harmala*), 230µgµL^{-1} and 315µgµL^{-1} (*M. pulegium*) and 680µgµL^{-1} and 800µgµL^{-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, isomenthone 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 bio-pharmaceuticals.

Conflict of Interests

Authors declare they have no conflict of interests.

Acknowledgments

This study was conducted at Institute of Microbiology, Azerbaijan National Academy of Sciences (AMEA).

Authors' Contributions

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

Funding/Support

The present study was supported by Research Fund of Institute of Microbiology, Azerbaijan National Academy of Sciences (AMEA).

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How to cite this article: Ghasemi M, Atakishiyeva Y. Investigation of 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. Infection, Epidemiology and Medicine. 2016; 2(2): 12-14.