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Antibacterial Effects of Hydro-alcoholic Extract of Pennyroyal, Cinnamon and Rhubarb on *Klebsiella Pneumoniae* and *Staphylococcus Aureus*: an In-vitro Study

Running title: Antibacterial effects of hydro-alcoholic extract of pennyroyal, cinnamon and rhubarb

Abstract:

Background: Medicinal plants have been used for many different purposes in the past. These plants may produce antimicrobial metabolites that affect the natural growth of microorganisms. The aim of this research was to study the antibacterial properties of rhubarb, cinnamon and pennyroyal on *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Materials and Methods: In this study, the effect of hydro-alcoholic extract of pennyroyal, cinnamon and rhubarb on *Staphylococcus aureus* and *Klebsiella pneumoniae* was investigated after their collection and extraction process. The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of this extract on bacteria were also measured.

Results: The MIC of hydro alcoholic extract of pennyroyal was above 400 mg/ml for both *Klebsiella pneumoniae* and *Staphylococcus aureus*. Also the MBC has been reported 400 and 500 mg/dL, respectively. The MIC of cinnamon extract for *Klebsiella pneumoniae* and *Staphylococcus aureus* were reported 250 and higher and 500 and higher, respectively. Also, the MBC was reported 500 and 600 mg/ml, respectively. *Klebsiella pneumoniae* was resistant to rhubarb plant extract, and the MIC and the MBC for *Staphylococcus aureus* were reported 200 mg/ml and higher and 400 mg/ml and higher, respectively.

Conclusion: The results of this study demonstrated that the hydro alcoholic extract of cinnamon, pennyroyal and rhubarb has a bacterial effect against *Klebsiella pneumoniae* and *Staphylococcus aureus*.

Key Words: Pennyroyal, Cinnamon, Rhubarb, *Klebsiella pneumoniae*, *Staphylococcus aureus*

Introduction :

The bacteria that cause infection in the urinary system are located in the intestine, anal areas and perineum. *Escherichia coli* (80% of cases), *Proteus*, *Pseudomonas*, *Klebsiella*, *Staphylococcus aureus*, and Coagulase Negative *Staphylococcus* are considered as the cause of these infections [1]. In *staphylococcus* genus, *Staphylococcus aureus* species, that exists on skin and everywhere such as respiratory system, is considered to be one of the most important pathogens in hospital and outpatient infections [2]. The emergence of resistant strains has caused many problems in the treatment of infections caused by pathogen SA (*Staphylococcus aureus*). Over the past twenty years, Methicillin Resistant *Staphylococcus aureus* (MRSA) strains have increased in different regions of the world [3]. *Staphylococcus aureus* is a gram-positive cocci, which is one of the most important factors in hospital infections and is found in various diseases such as boil skin disease and toxic shock syndrome, endocarditis and osteomyelitis, etc. This bacterium is fast resistant to antibiotics and can simultaneously withstand several antibiotics [4]. *Klebsiella* is a gram-negative, rod-shaped bacterium that is non-motile and has a polysaccharide capsule that covers the entire cell surface and creates resistance against many of

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the host's defense mechanisms. *Klebsiella* is a causative agent of pneumonia and also urinary tract infection [5].

The antimicrobial properties of herbal essential oils have been proven against a wide range of microbes, including bacteria, yeasts, and molds. Rhubarb with the scientific name "*Rheum ribes*" is a plant that is renowned as an antimicrobial drug in the traditional medicine. The extract of this plant in the laboratory has prevented the growth or elimination of microorganisms [6]. The oregano plant is a member of the *Lamiaceae* family and is a perennial herb. This genus includes more than 25 species and grows as a wild plant in the humid areas of the central and southern parts of Europe, southwest Asia and North Africa. The therapeutic properties of this herb have been proven in eliminating digestive disorders, vomiting, anorexia, ulcerative colitis, and liver disorders. The antimicrobial and antioxidant properties of several species of this plant are also well characterized [7].

Cinnamon, scientifically known as *Cinnamomum spp*, is a herb whose stems and young branches, as well as the oil from its leaves, have therapeutic applications. Cinnamon has mucilage, tannin, sugar, resin and essential oil; the essence of cinnamon is the most important part, and is found especially in the bark of the trunk. The major part of this essential oil is cinnamate aldehyde. In addition, the antibacterial effect of cinnamon has been considered and proven in many studies [8].

Treatment of disease can be improved through, the ethnomedicine and the elaboration of new agents. Prior to the establishment of antimicrobials and antioxidants agents derived from plants, the knowledge of traditional medicine needs scientific studies to validate the characteristics of medicinal plants. Considering the importance of studies against pathogenic bacteria and considering the antibacterial properties of these plants less attention was paid to studies, hence this study was designed [8]. The aim of this study was to investigate the antimicrobial effect of hydroalcoholic extract of rhubarb, pennyroyal, and cinnamon, on the growth of *Klebsiella pneumoniae* and *Staphylococcus aureus*.

2. **Materials** and methods:

2.1. **Chemicals** and **Reagents**

Dimethyl sulfoxide (DMSO) (Merck, cat no.: 472301), Alcohol, Blood Agar (Merck, cat no.:70133), Muller Hinton broth (Mueller-Hinton Broth |Sisco Research Laboratories cat no.: 49550 (SRL) (India)), 0.5 Whatman filter paper (Sigma)

2.2. Plant collection

In this experimental study, pennyroyal, cinnamon and rhubarb plants were collected from different regions of Chaharmahal and Bakhtiari province, and then identified and verified by a botanist (Dr. Shirmardi) at the Research Center of the Agriculture Jihad Organization of Chaharmahal and Bakhtiari province.

2.3. Extraction

The plants were dried and milled. Extraction was done by maceration method, that is, 100g of the plant in combination with 80% alcohol in 1-liter volume was kept for 72 hours in a dark environment. The obtained liquid was placed in a rotary machine (vacuum distillation) after filtration with 0.5 Whatman filter paper twice. It was then collected and placed at 37°C incubator. Finally the extract with honey consistency was obtained and a stock solution was prepared using Dimethyl sulfoxide (DMSO).

2.4. Preparation of standard bacterial strain

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The *Staphylococcus aureus* standard strain with ATCC 25923 and *Klebsiella pneumoniae* with ATCC 700603 and *Escherichia coli* with 25922 ATCC were prepared in Lyophilized form from Iranian Research Organization for Science and Technology

2.5. Preparation of Microbial Suspension

To prepare a microbial suspension which is equivalent to 0.5 McFarland standard CFU/ml, a 24 hour culture of bacteria was prepared on Blood Agar and then a suspension with 0.5 McFarland opacity was prepared in normal saline.

2.6. Determination of the MIC and MBC

The experiment of MIC was performed in sterile 96-well plates by micro-broth dilution method. An amount of 1000 μ l of 0.5 McFarland suspension diluted 1:75 to a well with 1810×1.5 CFU/ml bacteria equivalent to 5000 μ l from concentrations between 2 and 75 μ g/ml were added to the Muller Hinton broth medium. The microbial suspension and the culture medium were poured into one control row and the concentrations of the extracts were poured in another row.

The microplates were placed in a 37° C incubator for 24 hours, after that the lowest concentration of extract which had no turbidity due to the arrest in growth of bacteria, was considered as the Minimum Inhibitory Concentration [9].

To determine MBC, all the wells without turbidity were cultured on blood agar medium separately and incubated at 37 ° C for 24 hours and the lowest concentration of the extract in which the bacterium was not able to grow determined as MBC.

The MIC and MBC determining experiments were conducted 3 times for each extract [10].

The negative control of a well containing 106 μ l of non-extracted culture medium and 5 μ l of microbial suspension was considered.

The positive control of the different concentrations of ciprofloxacin plus the Muller-Hinton Bruth culture medium and 5 μ l of bacterial suspension were considered.

Results:

Klebsiella pneumoniae was susceptible to 400 μ g / ml of pennyroyal and 250 mg / ml of cinnamon, but the susceptibility of this bacterium to rhubarb was higher than 600 mg /ml concentration. The activity of *Staphylococcus aureus* was stopped at 400, 500 and 200 mg / ml concentrations of pennyroyal, cinnamon and rhubarb, respectively (Table 1).

On the other hand, the minimum bactericidal concentration (MBC)for *Staphylococcus aureus* to pennyroyal, cinnamon and rhubarb has been calculated 600, higher than 600 and 400 mg / ml respectively, and also the *Klebsiella pneumoniae* bacteria were completely eliminated by pennyroyal, cinnamon and rhubarb at 600, 500 and higher than 600 mg / ml concentrations, respectively (Table 1).

The sign (+) indicates the lack of microorganisms growth on the culture medium and the presence of antibacterial activity of the hydroalcoholic extract of the savory plant. Sign (-) indicates the growth of microorganism on the culture medium and the absence of antibacterial activity of the hydroalcoholic extract of the savory plant (Table 1).

The sign (+) indicates the lack of microorganisms growth on the culture medium and the presence of antibacterial activity of the hydroalcoholic extract of the cinnamon plant. Sign (-) indicates the growth of

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microorganism on the culture medium and the absence of antibacterial activity of the hydroalcoholic extract of the cinnamon plant (Table 1).

Discussion:

Increasing resistance to excessive use of synthetic antibiotics found that alternative drugs that have both antibacterial and anti-inflammatory effects should be considered. Many studies have highlighted the anti-microbial effects of plants with medicinal properties. Antibacterial properties of many plants are available in various studies on infections and infectious diseases. Also the results of the current study on the antibacterial properties of pennyroyal, cinnamon and rhubarb herbs demonstrated that all these three plants have antibacterial properties against *Klebsiella pneumoniae* and *Staphylococcus aureus*. *Staphylococcus aureus* is one of the five most common causes of hospital infections, especially postoperative wound infections. Each year, 500,000 people in US hospitals become infected with *Staphylococcus aureus* [11]. The major bacteria responsible for urinary tract infections are intestinal bacteria. *Klebsiella pneumoniae* is one of the opportunistic pathogens that causes hospital infections. The prevalence of extended-spectrum beta-lactamases in *Klebsiella pneumoniae* strains leads to the spread of antibiotic resistance and mortality in patients [12].

Klebsiella pneumoniae typically causes bronchopneumonia and bronchitis. In these patients, the risk of pulmonary abscess (empyema) is increased. Even with an antibiotic, it has 50% mortality rate, and mortality for those who have alcoholism or bacteremia reaches 100%. This opportunistic pathogen can also cause chronic pulmonary disease, nasal epithelium atrophy and rhinoscleroma. Recently, the antibiotic resistance of the *Klebsiella pneumoniae* strains has prevailed [13].

Due to the lack of similar studies that worked exactly on the bacteria and plants we studied, we had to use studies that have been working on the antibacterial effects of the plants studied on other bacteria.

Lei Guo et al. demonstrated that rhubarb and its compounds have antibacterial effects against *Vibrio harveyi*. In this study, the lowest dose with the MIC was reported 0.25 mg / dL [14]. In the current study, the antibacterial effects of rhubarb on *Staphylococcus aureus* were detected, whereas no antibacterial effects against *Klebsiella pneumoniae* were observed. However, the reported dosage was significantly higher in the current study compared to the one by Lei Guo at 400 mg / ml.

A study by Nassan et al. showed that cinnamon has antibacterial effects against *Staphylococcus aureus* at a dose of 3-4 mg / dL [15]. The findings of current research, consistent with the study of Nassan et al., showed the antibacterial effects of cinnamon against *Staphylococcus aureus* at concentrations of 500 mg / ml.

Wang et al. studied the effect of *Cinnamomum zeylanicum* and the main aldehyde (Cinnamaldehyde) against *Porphyromonas gingivalis*, and their results showed that the aldehyde within cinnamon, as an antibacterial agent, can be effective for its inhibitory effect on *P. gingivalis* bacterium [16]. The results of our study is also in accordance with previous research and has proven the antibacterial effect of cinnamon against *Staphylococcus aureus*.

Syahdiana Waty et al. studied the antibacterial effect of hydroalcoholic extract of cinnamon on *Streptococcus* bacteria. Eight species of *Streptococcus* sp. including *S. sanguinis*, *S. salivarius*, *S. pluranimalium*, *S. pneumoniae*, *S. alactolyticus*, *Kocuria rosea*, *Kocuria kristinae*, and *Spingimonas paucimolis* were studied and the results showed that ethanolic extract of cinnamon bark has significantly

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inhibited the growth of *Streptococcus*. The results of the present study are consistent with the study by Syahdiana Waty, confirming the antibacterial properties of cinnamon[17].

In the research by Chunxia et al., the antibacterial properties of raw extract of rhubarb and its main compounds against *Aeromonas hydrophila* were studied. The results showed that anthraquinones (anodyne) isolated from the rhubarb, after penetration into the membrane through binding to the DNA, causes the death of the cells [18].

Our study and such other studies has some limitations. One of the limitations of our study was the lack of molecular studies to determine the pathway to the effect of these herbal medicines on stopping the growth of the bacteria in question. Another limitation of the study was the lack of time-dependent evaluation of herbal drugs in doses.

Conclusion:

In general, this study showed that the studied medicinal herbs may have antibacterial effects on *Staphylococcus aureus* and *Klebsiella pneumoniae*. However, further experiments are necessary to study other extracts of these plants and purify the important compounds, as well as the use of different bacterial strains.

Ethical approval and consent are not applicable.

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Table 1: MIC and MBC at different concentrations of pennyroyal, Cinnamon, Rhubarb extract

Organism	Pennyroyal extract concentration(mg/ml)								
	100	150	200	250	300	400	500	600	Control
<i>Klebsiella pneumoniae</i>	-	-	-	-	-	+	+	+	-
<i>Staphylococcus aureus</i>	-	-	-	-	-	+	+	+	-
MBC	Pennyroyal extract concentration(mg/ml)								
	100	150	200	250	300	400	500	600	Control
<i>Klebsiella pneumoniae</i>	-	-	-	-	-	-	-	-	+
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	-	-	+
Organism	Cinnamon extract concentration(mg/ml)								
	100	150	200	250	300	400	500	600	Control
<i>Klebsiella pneumoniae</i>	-	-	-	+	+	+	+	+	-
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	+	+	-
MBC	Cinnamon extract concentration(mg/ml)								
	100	150	200	250	300	400	500	600	Control
<i>Klebsiella pneumoniae</i>	-	-	-	-	-	-	+	+	-
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	-	+	-
Organism	Rhubarb extract concentration(mg/ml)								
	100	150	200	250	300	400	500	600	Control
<i>Klebsiella pneumoniae</i>	-	-	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i>	-	-	+	+	+	+	+	+	-
MBC	Rhubarb extract concentration(mg/ml)								
	100	150	200	250	300	400	500	600	Control
<i>Klebsiella pneumoniae</i>	-	-	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i>	-	-	-	-	-	+	+	+	-