

Antibacterial Activity of Alcoholic Extracts of Two *Clematis* L. (Ranunculaceae) Species from Iran

Raei F (Ph.D.)¹, Ghorbani Nohooji M (Ph.D.)^{2*}, Habibi M (M.Sc.)³, Ashoori N (M.Sc.)¹

1- Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University GC, Tehran, Iran

2- Cultivation and Development Department of Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran

3- Biology Department, Faculty of Basic Sciences and Engineering, Gonbad University, Gonbad'e Kavous, Iran

*Corresponding author: Institute of Medicinal Plants, ACECR, P.O.Box: 31375-369, Karaj, Iran

Tel: +98-26-34764010-20, Fax: +98-26-34764021

Email: m.gh.nahooji@gmail.com, m.ghorbani@acecr.ac.ir

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Abstract

Background: Despite the discovery of numerous antibiotics, drug resistance still remains as a major problem. Therefore, it is important to introduce and replace new sources of drug such as medicinal plants with antimicrobial properties.

Objective: The genus *Clematis* (Ranunculaceae family) has six species in Iran. Aerial parts of *Clematis* have been used to cure fever and chronic diseases such as Rheumatism in traditional medicine. In this research, antibacterial activity of ethanolic and methanolic extracts of aerial part in two major species of the genus in Iran (contains *C. orientalis* and *C. ispahamica*) were studied.

Methods: Aerial plant parts were dried in shade, powderized and then ethanolic and methanolic extracts were prepared. Antibacterial activity of the two extracts was measured against six laboratory standard strains including gram positive and gram negative bacteria by disc diffusion. Minimum inhibitory concentrations were also determined using broth microdilution.

Results: Results showed that antibacterial activity of methanolic extracts is stronger than the ethanolic one. Also, *C. ispahamica* has stronger antibacterial activity in comparison to *C. orientalis*. Gram positive test bacteria showed the most susceptibility to both extracts compared to gram negative organisms.

Conclusion: According to antibacterial effects of alcoholic extracts, it seems necessary to diagnose effective antibacterial components to compare results with existed finding and introduce a new trapeutic source.

Keywords: *Clematis ispahamica*, *Clematis orientalis*, Aerial part extracts, Antibacterial activity

Introduction

Nowadays, despite the discovery of various antibiotics, the problem of microbial resistance has still remained and is increasing. Thus, extensive researches are required to discover new drugs and replacing them with chemical medications [1, 2]. Iran is a vast country, with its diverse climate whose plant's biodiversity is very rich. More than 8000 plant species showing representatives from many plant families were scattered in Iran. Also the unique climatic variations have provided possibility of growing many plants of other parts of the world in this country [2, 3, 4, 5, 6].

Among these medicinal plants genus *Clematis* L. belongs to the buttercup family (Ranunculaceae) and with 250-300 species in the world, it is considered one of the largest genus of this family. Many species of this genus are found in the temperate regions of Europe and Asia as well as at high tropical areas [7]. Because of having different pharmaceutical compounds including glycosides, saponins and alkaloids, different species of this genus are known as a plant widely used in traditional medicine; therefore it has significant economic and medicinal importance [7]. In Pakistan and Kashmir some species of this plant are used as a food preservative and also to eliminate of skin damages caused by Leprosy disease [8]. As reported some species of this genus are used to remove skin diseases, relieve itching skin, improve wound healing, cope with viral fever and relieve cardiac arrhythmias in the Himalayan regions and India. Antibacterial effects of this plant have also been reported in many of these areas [9, 10]. In other parts of the world similar studies have been done for a few species of this genus. All of them have shown wide and variable range of extract

effects on bacterial pathogens [11, 12]. In addition, numerous studies have been conducted on different species of *Clematis* in East Asia which showed that extract of this plant had a significant effect as an antimicrobial, diuretic and anti-inflammation agent [13, 14, 15]. In another study it was noted that 26 species of *Clematis* are used for the treatment of chronic diseases such as neurological disorders, syphilis, malaria, rheumatism, gout, diarrhea and asthma in Europe and East Asia. Several properties have also been mentioned for this plant, including pain relief, anti-inflammatory, diuretic, anti-tumor and anti-cancer effects [16]. Similarly for the triterpene glycosides isolated from *C. ganpiniana* have demonstrated cytotoxic effects on several cancer cell lines [17]. According to Flora Iranica treatment, 6 species of this genus are grown in Iran from which *C. orientalis* L. and *C. ispahamica* Boiss. have wide dispersion and so these two species are used by people more than the other species [18].

Since no study has been done about the antimicrobial effects of Iranian species of *Clematis* and due to the importance of pharmacological effects of it, in this study the antimicrobial effects of the two mentioned species having wide distribution in Iran has been studied.

Materials and Methods

Plant material and preparation of plant extracts

Plant specimens were collected from a region around the Azad-shahr city in the Golestan province in Iran at August 2012. After accurate identification of specimens collected using botanical reliable sources [19,



20], herbarium specimens were prepared and deposited in the SBUH (Shahid Beheshti University Herbarium) with 8400275 and 8500083 herbarium numbers for *C. orientalis* and *C. ispahanica* respectively. Plant materials were dried at room temperature in the shade, and then aerial parts (Consisting delicate stems, foliage and flowers) were powdered by an electric blender, 10 gram of each of the powdered materials were macerated with 100 mL of methanol and ethanol 96% for 48 hours and mixture of crushed plant powder and solvent were stirred every few hours. Finally after filtration, the extracts were concentrated and dried. The condensed products were weighted and kept at 4 °C prior to test [21, 22].

Bacterial strains

In this study six Gram positive and Gram negative laboratory standard bacteria strains including *Bacillus subtilis* (ATCC 465), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031) and *Pseudomonas aeruginosa* (ATCC 85327) were examined.

Antibacterial susceptibility testing

The antibacterial activity of the alcoholic extracts was determined by disc diffusion method. To prepare the discs containing the extracts, 25 µl of each extract (12 mg/disc) were added to sterile 6 mm discs. Also the discs containing solvent were prepared as controls and all discs were dried at room temperature.

By using Kirby-Bauer method [23], 4-6 colonies of fresh culture of each strain were transferred to 5 ml of Muller-Hinton broth (MHB, Liofilchem, Italy), the tubes were incubated at 37°C for 4-6 hours, and then tubes turbidity were compared and matched to 0.5

McFarland standard corresponds to around 10⁸ cells/ml. In next step, 0.1 ml of each suspension was spread on a Mueller-Hinton agar (MHA, Liofilchem, Italy) plates by sterile swab, and discs containing both alcoholic extracts were placed on the microbial lawns, antibiotic disc including penicillin (10U, PadtanTeb, Iran) and control discs were also included. The plates were incubated at 37°C for 18-24 h. The diameter of inhibition zones were measured following the incubation period and reported in mm. Also the tests were carried out in triplicate [21, 22, 23].

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) values were determined by broth micro-dilution as recommended by Clinical and Laboratory Standards Institute (CLSI) [24]. Serial two-fold dilutions of the alcoholic extracts within the range of 1-25 mg/ml were made in MHB. In high concentrations the extracts were solved using ultrasonic for less than 40 seconds. Serial dilutions of each extract were individually placed in 96-well microtiter plates. All wells were filled with 50µl of MHB. Fifty µl of 10% extracts were added to the first wells and after mixing MHB and extract, 50µl from first wells were transferred to second well. This procedure was repeated for solutions in wells 2 to 9 and 50µl were taken out from ninth wells. Rest of the wells considered for controls of bacteria, MHB and alcoholic extracts alone. Fifty µl of fresh bacterial suspensions, prepared from overnight grown cultures in MHB were added to give a final concentration of 10⁶ organisms/ml. The microplates were incubated at 37°C for 24-28 h. After incubation, at least dilution of each row with no growth was recorded as MIC. The tests were repeated in triplicate [22, 23, 24].

Results

The results of the antibacterial activity of alcoholic extracts by the disc diffusion assay are compared and given in Table 1. The experiments were performed in triplicate, and the results are presented as mean values of the three measurements. The results of the antibacterial activity of ethanolic and methanolic extracts of *C. orientalis* and *C. ispahanica* using disc diffusion on mentioned bacterial strains, it was shown the maximum inhibitory effect can be seen on Gram positive bacteria *B. subtilis*, *S. aureus* and *E. faecalis*. Gram positive test bacteria showed the most susceptibility to both extracts compared to gram negative organisms, as *K. pneumoniae* and *E. coli* showed little sensitivity with 9 and 10 mm zone diameter respectively. Results obtained from the controls indicated that solvents had no effect on the microorganisms.

The MIC values are compared in Table 2. Results obtained from the MIC of alcoholic extracts have been variable between 3.125 -25 mg/ml. Gram positive bacteria such as *S. aureus* with a MIC of 3.125 mg/ml was the most sensitive bacteria were tested against extracts and then the bacteria *Bacillus* and *Enterococcus* can be noted respectively. As shown in the Tables 1 and 2, the best results in the case of gram positive bacteria is obtained for *S. aureus* and *B. subtilis* with 10 - 14.6 and 13.3 - 14.3 mm zone diameters and MIC values between 3.125 - 6.35 and 6.25 mg/ml. Ethanolic extract of *C. ispahanica* was more effective than other extracts on cultured *E. coli*. In generally, results showed that antibacterial activity of methanolic extracts is stronger than the ethanolic ones. Also, *C. ispahanica* has stronger antibacterial activity in comparison to *C. orientalis*.

Table 1- The results of the antibacterial activity of alcoholic extracts by the disc diffusion assay.

Bacteria	<i>C. orientalis</i>		<i>C. ispahanica</i>		Penicillin
	Methanolic extract	Ethanolic extract	Methanolic extract	Ethanolic extract	
1 <i>Staphylococcus aureus</i>	11 ± 1.52	10 ± 1	14.6 ± 2.08	11 ± 0.57	21
2 <i>Bacillus subtilis</i>	14.3 ± 1.52	13.3 ± 1.52	14 ± 1	14.3 ± 2.08	20
3 <i>Enterococcus faecalis</i>	10.3 ± 1.52	9.6 ± 1.52	12.3 ± 2.08	10.3 ± 1.52	23
4 <i>Escherichia coli</i>	10.3 ± 1.52	10.3 ± 1.52	11.3 ± 1.15	12 ± 1	20
5 <i>Klebsiella pneumoniae</i>	9.3 ± 0.57	8.6 ± 0.57	10 ± 1	8.6 ± 0.57	17
6 <i>Pseudomonas aeruginosa</i>	9.6 ± 0.57	10 ± 1.14	9.6 ± 1.52	8.6 ± 1.15	-

Table 2 - Minimum inhibitory concentration results

Bacteria	<i>C. orientalis</i>		<i>C. isphahanica</i>	
	Methanolic extract	Ethanollic extract	Methanolic extract	Ethanollic extract
1 <i>Staphylococcus aureus</i>	3.125	6.25	3.125	3.125
2 <i>Bacillus subtilis</i>	6.25	12.5	6.25	6.25
3 <i>Enterococcus faecalis</i>	6.25	12.5	6.25	12.5
4 <i>Escherichia coli</i>	>25	>25	25	25
5 <i>Klebsiella pneumoniae</i>	>25	>25	>25	>25
6 <i>Pseudomonas aeruginosa</i>	>25	>25	>25	>25

Discussion

Studies have shown that *Clematis* species have various compounds including triterpenes, flavonoids, lignins, coumarins, alkaloids, organic acids, macrocyclic compounds and poly phenols extensively. Among these compounds, the triterpenes, saponins, flavonoids and lignins are found more abundantly than other groups [16]. Due to this reason, ethanolic and methanolic extracts of the two species *C. isphahanica* and *C. orientalis* which are scattered in different parts of Iran, have been studied. So far, no report has been provided about their antimicrobial and medicinal properties. Sporadically researches on many species of this genus have been done around the world representing a wide range of effects of these plants on the growth of bacterial agents. Similarities and differences can be observed in the results of this study and other studies. For example a study has shown that the ethanolic extract of *C. papuasica* against bacteria such as *Staphylococcus*, *Enterococcus* and *E. coli* had considerably antimicrobial effects, but *Pseudomonas* and *Klebsiella* were not sensitive to these extracts [11]. In the results of the present study,

ethanolic and methanolic extracts of the two species showed the same antimicrobial effect on *Staphylococcus*, *Enterococcus* and *E. coli*, whereas a weak antimicrobial effect against *Pseudomonas* and *Klebsiella* was shown.

In the same way in separate studies [9, 10] it was expressed that alcoholic extracts of species of the genus *Clematis* have the greatest impact on bacteria, including *Staphylococcus* and *Klebsiella*, while these results are different from the results obtained in this study.

The methanolic extract of the leaves of *C. hirsuta* is also a powerful antifungal agent against *Candida albicans* and dermatophytes such as *Trichophyton*, *Epidermophyton* and *Microsporum*. Antibacterial properties of this plant had the same inhibitory effect on the Gram positive bacteria, *Staphylococcus aureus*, and *Mycobacterium frutitium* and significant antimicrobial effects on Gram-negative bacteria such as *Salmonella*, *Enterobacter*, and *Proteus* [12]. The results of the present study were similar to some parts of the mentioned results and they were inconsistent with some other parts.

Because of the wide variety of species of this genus in the world, these differences are



caused by many factors, including differences in climate condition of growing plants, genetic and epigenetic differences between various species, different seasons and use of different plant parts. Results of this study show the effectiveness of ethanolic and methanolic extracts of *C. orientalis* and *C. ispahanica* for the treatment of infections caused by bacteria for major clinical pathogens such as *S. aureus*. During the past decade, this bacterium has developed resistance to many commonly used antibiotics [25]. In this study, the extracts of *Clematis* showed activity against *S. aureus* and can be used as raw materials for phytotherapy.

Conclusion

Ultimately it can be stated that according to therapeutic uses of genus *Clematis* and

because its ethanolic extracts possess antimicrobial effects (especially on Gram positive bacteria) it is necessary to do extensive researches on various species of this genus for achieving an accurate detection and isolation of antimicrobial active ingredients and introducing sources of new drugs. According to the importance of these medicinal plants, further studies are needed to determine the toxicity of the extracts before their internal use and antibacterial activity of *Clematis* essential oil.

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