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Original Article

Phytochemical Profile and Antibacterial-AntiQuorum Sensing Properties of *Citrus medica L.*

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Abstract

Introduction: Natural resources are becoming more and more important as the need to find solutions to the antibiotic resistance growing crisis. The assessment of medicinal plants' antibacterial and antiquorum-sensing properties is gaining popularity in this field of research every day. The study reported here aimed to investigate the inhibitory activity of the methanolic extract of *Citrus medica* L. on the inhibition of violacein pigment production in *Chromobacterium violaceum* ATCC 12472 and some virulence factors in *Pseudomonas aeruginosa* PAO1. Additionally the phenolic content of the extract was also determined by HPLC analysis.

Methods: The phytochemical content of the plant extract was determined and its antibacterial activity on some bacteria was tested. Also antibiofilm effect on PAO1 was determined, and violasin pigment inhibition on *C. violaceum* was investigated.

Results: It was observed that the methanolic extract had an inhibition effect of 32% on violacein pigment production and a strong inhibition effect of 88% on biofilm formation caused by PAO1. According to the results of the phytochemical content analysis, benzoic acid was determined as the major component of the extract with a concentration value of 41.9 µg/mL.

Conclusion: *Citrus medica* L, like many plants, has antibacterial and antiquorum sensing activity and may be a potential agent in the fight against infectious diseases.

Keywords: Antiquorum sensing, Citrus, phytochemical, PAO1, violacein

1. Introduction

Long-term use of antibiotics in the treatment of bacterial infections causes some undesirable side effects and infections resistant to antibiotic treatment. Resistance is not limited to one drug, but is seen in many drugs and appears as multiple antibiotic resistance. This prolongs the duration of treatment and increases the length of hospitalization. It also increases the investment and cost of health services (1).

The fact that all these problems also threaten public health pushed scientists to search for different solutions, especially natural solutions of plant origin (2). Plants generally have antioxidant effect

and capacity against free radicals that can cause irreversible damage to important compounds such as carbohydrates, lipids, proteins, nucleic acids and DNA in our body. Plants show this effect by preventing the formation of reactive oxygen species, neutralizing free radicals and detoxifying radicals by converting them into more harmless compounds (3). Phenolic compounds found in the roots, leaves and above-ground tissues of plants used for many purposes among the people, which provide many properties such as antimicrobial, anti-inflammatory and anticancer, and the components in their essential oils. The determination of the existence of plant ingredients on scientific grounds has increased the trust in plants and is seen as one of the options that can be evaluated in the fight against infectious diseases. In addition, another option is to prevent bacteria from communicating with other bacteria, which is different from the antibacterial effect (4). The discovery that many pathogenic bacteria produce virulence factors that are effective in the development of successful infection process through the quorum sensing (QS) system has made this system a therapeutic target for the design and development of a new class of drugs that potentially control pathogenicity (5).

The QS mechanism depends on the synthesis, release and uptake of autoinducers (AIs) in the surrounding environment, the concentration of which is related to the density of secreting bacteria. AIs, extracellular signaling molecules that accumulate in the environment in proportion to cell density, are used for this intercellular communication. Their function is to regulate gene expression in other cells of the community and control a range of bacterial responses. QS systems have been present in bacteria since time immemorial, and since ancient times bacteria have regulated a variety of cellular functions through their QS machinery, including luminescence, biofilm formation, sporulation, development of genetic competence, synthesis of peptide antibiotics, production of secreted proteolytic enzymes, virulence factor expression, pigment production, plant-microbe interactions and motility (6). Disruption of this communication system or bacterial QS activity leads to attenuation of the microbial virulence.

Inactivation or disruption of QS signaling molecules is known as QS inhibition or quorum quenching. This inhibition can be achieved by various means, such as the development of antibodies against QS signaling molecules, enzymatic degradation of QS signaling molecules or agents that block QS. These strategies interfere with the cell-to-cell communication system and monitor the growth of infectious bacteria without stopping them, thus preventing the development of antibiotic resistance (7).

Medicinal plants have been investigated for their therapeutic value in traditional medical practice and it has been proven that medicinal plants are the natural source of compounds that can be used against many diseases (8). Many plants contain a wide variety of chemicals that have important biological effects on humans (9). Bioactive compounds that develop as a result of secondary metabolic activities of plants, which cannot be consumed as food but have beneficial effects for human health are called 'phytochemicals' (10). These compounds prevent degenerative diseases, act as antiallergenic, anti-inflammatory, antimicrobial, antithrombotic and vasodilator agents (11). The main compounds responsible for the antimicrobial effect in plants are known as phenolics, phenolic acids, quinolones, saponins, flavonoids, tannins, coumarins, terpenoids and alkaloids (12). Phenolic substances constitute the most important groups of natural antioxidants. In addition to their antioxidant activities, they chelate metal ions and inhibit transcription factors that initiate and support tumor development by stimulating detoxifying enzymes (9). In this study, antibacterial and anti-quorum sensing properties of *Citrus medica* L. plant were investigated and some phenolic compounds were determined by HPLC.

2. Methods

2.1. Plant Extraction

The peel of *C. medica* L. was dried in ~~the~~ shade at room temperature away from direct sunlight. It was powdered with the help of a steel blender

(Waring 8011 EB) and 10 g was weighed and 100 mL of solvent (methanol) was added. The solvent-powder mixture was kept in an ultrasonic water bath for half an hour, and the methanol-tree melon mixture taken from the ultrasonic water bath was filtered through coarse filter paper. The plant extract remaining in the flask whose solvent was completely removed in a rotary evaporator (Heidolph Hei-Vap Rotary Evaporator) under vacuum at 40-45ºC was weighed and recorded. All of the plant in the flask was extracted with dimethylsulfoxide (DMSO). It was stored in the refrigerator at +4ºC.

2.2. High-Performance Liquid Chromatography (HPLC) Analysis

The *C. medica* L. extract's phytochemical composition was assessed using the High-Performance Liquid Chromatography (HPLC) method (13). Table 1 displays the HPLC analytical process.

Table 1. Conditions of chromatography

	Time (min)	A (%)	B (%)
Detector: Photo Diode Array Detector (λ max. 278 nm)	0	93	7
	20	72	28
Autosampler: SIL–10AD vp	28	75	25
System controller: SCL-10A vp	35	70	30
Pump: LC-10AD vp	50	70	30
Degasser: DGU-14a	60	67	33
Column heater: CTO-10 A vp	62	58	42
Column: Agilent Eclipse XDB C-18 (250 mm × 4.6 mm), 5 µm	70	50	50
Column temperature: 30 °C	73	30	70
Mobile phases: A: acetic acid– water (3:97 v/v), B: methanol	75	20	80
Flow rate: 0.8 mL/ dak.	80	0	100
İnjection volume: 20 µL	81	93	7

2.3. Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Test

The antibacterial activity of *C. medica* L. was tested on Gram positive (*Bacillus cereus* ATCC 11778, *Enterecoccus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923) and Gram negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* PAO1) bacteria and its antiquorum sensing effect was tested on *P. aeruginosa* PAO1 and *Chromobacterium violaceum* (CV12472) bacteria. In this method, 96-well microplates were prepared. In the wells containing Mueller Hinton Broth medium, 100 µL of plant extract was added and two-fold serial dilutions were made respectively. 5µL of bacterial suspension adjusted to 0.5 McFarland turbidity was added and the microplates were incubated overnight at 30/37°C. After incubation, the microplates were evaluated and the lowest concentration without growth was determined as the MIC value. Experiments were performed in 3 replicates. MBC test confirmed by point-inoculating MH media from wells for each micro-organism tested (14).

2.4. Pyocyanin Inhibition Test

To the LB medium containing *C. medica* L. to be tested for its effect on pyocyanin pigment production, 100 µL of PAO1 bacterial culture with OD 0.05 at 600 nm was added and incubated at 37°C for 16-18 hours with shaking. Then 5 mL of chloroform was added to PAO1 cultures grown in LB medium and vortexed for 45 seconds. The phase separated from chloroform at the bottom of the bottles was separated into glass tubes as 2 mL. Then 1 mL of 0.2 M HCl-water was added to the glass tubes and vortexed for 45 seconds and the pink phase formed at the top of the tubes was read at OD 520 nm and recorded. The experiment was performed in 3 replicates (15).

2.5. Biofilm Inhibition Test

Luria Bertani Broth (LBB) medium, 20 µL *C. medica* L. and 5 µL PAO1 bacteria equivalent to 0.5 McFarland turbidity were added to 96 microplates and the microplate was incubated for 48 hours. After 48 hours, the contents were poured out and washed three times with distilled water, 0.1% crystal violet was added to the wells as 200 µL and waited for 30 minutes. After waiting, the crystal violet was poured and washed three times

with distilled water. 200 µL of 95% EtOH was added to each well and waited for another 15 min. The results were read at OD 570 nm, compared with the control and recorded. The experiment was performed in 3 replicates (16).

2.6. Violacein (*C. violaceum* 12472) Pigment Inhibition Test

Overnight culture (10 µL) of *C. violaceum* (adjusted to 0.4 OD at 600 nm) was added to sterile microplates containing 200 µL. Microplates containing CV12472 and *C. medica* L. as positive control were incubated at 30 °C for 24 h and observed for the decrease in violacein pigment production. Absorbance was read at 585 nm. The experiments were performed in 3 replicates (17).

2.7. Statistical Analysis

This study was designed according to the "randomized plots experimental design" and three replicates were used in each treatment. The data were subjected to analysis of variance (ANOVA) with JUMP statistical package program and the differences between treatments were evaluated with LSD multiple comparison test.

3. Results

3.1. HPLC Results

The presence of protocatechic acid, catechin, epicatechin, sinapinic acid, benzoic acid, rutin, hesperidin, and cinnamic acid is demonstrated by the HPLC chromatogram of the methanolic extract of *C. medica* L. A sample chromatogram is displayed in Fig 1.

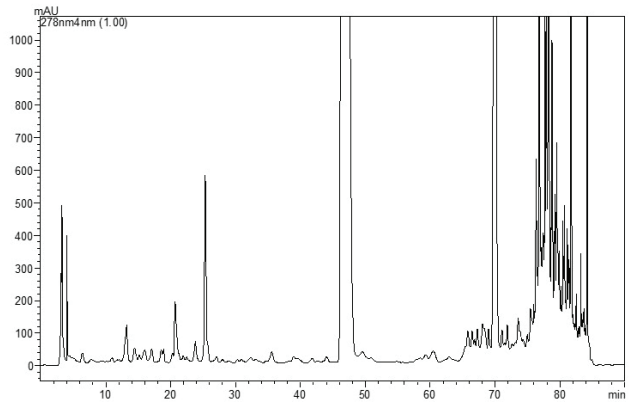

Figure 1. HPLC chromatogram for the main phenolic compounds identified in the methanolic extract of *C. medica* L.

During the chromatogram, the common spectrum containing the peak maximums of each analyte was studied at 278 nm. The highest concentration was found in benzoic acid as 41.9 µg/mL, which was followed by sinapinic acid as 7.6 µg/mL, cinnamic acid as 7.0 µg/mL (Table 2). Also, catechin as 6.3 µg/mL, hesperidin as 4.7 µg/mL, rutin as 3.5 µg/mL, epicatechin as 1.9 µg/mL, protocatechic acid as 0.5 µg/mL phytochemicals.

Table 2. Concentrations of the primary phenolic components found in *C. medica* L.'s methanolic extract.

Phytochemicals	Concentrations (µg/mL)	Retention time (min)
Protocatechic acid	0.5	9.0
Catechin	6.3	12.70
Epicatechin	1.9	19.7
Sinapinic acid	7.6	31.3
Benzoic acid	41.9	36.9
Rutin	3.5	46.1
Hesperidin	4.7	52.6
Cinnamic acid	7.0	68.3

3.2. MIC and MBC Test Results

The minimum inhibition concentration of *C. medica* L. was studied on Gram positive and Gram negative bacteria and the values are given in Table 3. According to the table, the best antibacterial effect of *C. medica* L. was observed on *C. violaceum* 12472. The weakest antibacterial effect was observed on *E. coli* and *S. aureus* bacteria.

Table 3. MIC and MBC results of *C. medica* L. peel extract

Microorganims	MIC (mg/mL)	MBC (mg/mL)
E. coli ATCC 25922	60	121
S. aureus ATCC 25923	60	121
P. aeruginosa PAO1	30	60
P. aeruginosa ATCC 27853	30	60
B. cereus ATCC 11778	30	60
C. violaceum CV 12472	15	30

3.3. Pyocyanin InhibitionTest

Many *P. aeruginosa* strains are soluble bacteria, giving the colonies a blue-green color pyocyanin, a phenazine-derived pigment has the ability to produce *P. aeruginosa* low molecular weight produced by pyocyanin molecule, which has

important is one of the pathogenicity factors. Respiratory pathways interrupted ciliary activity and oxidative and is responsible for neutrophil-associated tissue damage (18,19). Pyocyanin production test proved that *C. medica* L. inhibited pyocyanin pigment production in PAO1 by 20%. Although an average inhibition was observed, it was statistically significant ($p<0.01$) (Fig 2).

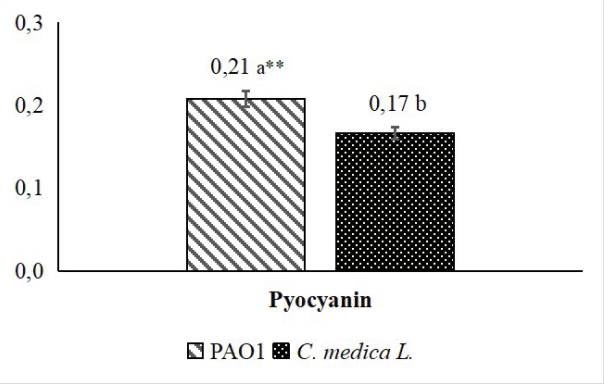

Figure 2. Inhibition effect of *C. medica* L. on pyocyanin production.**The difference between the means shown with different letters is statistically significant ($p<0.01$).

3.4. Biofilm Inhibition Test

According to test result of biofilm formation on PAO1, it was observed that *C. medica* L. had a high inhibitory effect of 88% on biofilm formation (Fig 3).

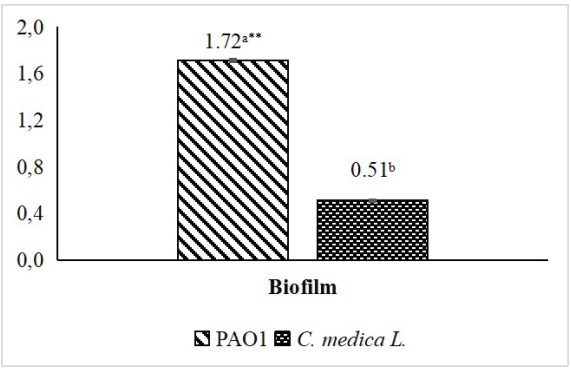

Figure 3. Inhibition effect of *C. medica* L. on biofilm formation. **The difference between the means shown with different letters is statistically significant ($p<0.01$).

3.5. Violacein Inhibition Test

In *C. violaceum*, the QS system is responsible for violacein (purple) pigment formation. In this study, *C. medica* L. inhibited violacein pigment formation by 32% (Fig 4).

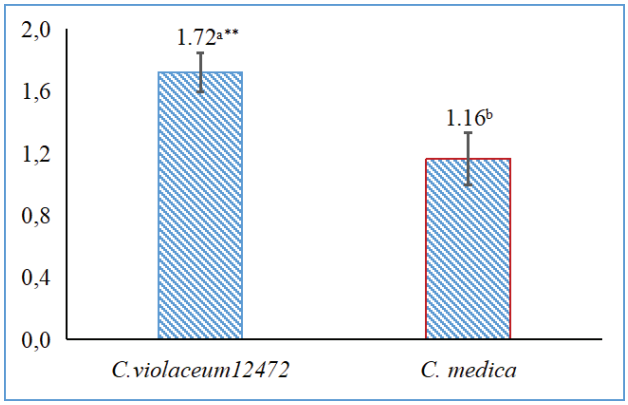

Figure 4. Inhibition effect of *C. medica* L. on violacein pigment formation. **The difference between the means shown with different letters is statistically significant ($p<0.01$).

4. Discussion

The need for novel antimicrobial agents is growing because bacteria can become resistant to existing medications. Medicinal plants are abundant in bioactive substances that have antibacterial properties. Since *C. medica* L. contains a large concentration of phytochemicals with antimicrobial characteristics, like citral, linalool, and limonene its antimicrobial properties are unquestionably the best studied of its various biological properties (20-22). The *C. medica* L. is the oldest wild product of the citrus family known to possess various pharmacological and nutraceutical properties. The presence of phytochemicals in the peel and leaves shows antioxidant effects that have a protective effect against many diseases such as diabetes, cancer, hypercholesterolemia and other chronic diseases caused by various oxidative stresses (23). In this study, antibacterial and antiquorum sensing properties of *C. medica* L. were investigated and as a result of the experiments, the presence of antibacterial effect of *C. medica* L. on various bacteria was demonstrated and MIC-MBC concentration were determined. While it was found to have a similar MIC value on Gram positive and Gram negative bacteria, the lowest MIC and MBC value was found on *C. violaceum* with a concentration of 30/60 mg/mL. In a study an ethanol extract from the exocarp of *C. medica* var. sarcodactylis was effective against *B. cereus* (MIC 2.5 mg/mL) than *E. coli* (MIC 10 mg/mL). The exocarp extract's increased antibacterial action could be explained

by its higher coumarin concentration (24). The main component of *C. medica* L. used in this study was found to be benzoic acid after hplc test and it has been reported in previous studies that the antimicrobial activity of benzoic acid is primarily against yeasts and molds (25).

The antibacterial properties of extracts from the roots, leaves, and bark of the *C. medica* species grown in Uttarakhand, India's Kumaun region were evaluated on a number of bacterial strains. The root and juice extracts exhibited the greatest activity, with inhibition zones of 19 nm and 17 mm, respectively. These values were even greater than those of the conventional medication, chloramphenicol, which has an inhibition zone of 14 mm (26). Sharma et al., on the other hand, reported that a *C. medica* L. juice extract did not affect the development of *B. subtilis, S. aureus*, *E. coli* or *K. pneumoniae* (27).

Plant contents may show annual changes depending on the region where they grow, rainfall rate and different climatic conditions (28). In this regard, Sun et al. found through a meta-analysis that the amounts of phenolic compounds in aromatic and medicinal plants rise in response to yearly rainfall declines and temperature rises (29).

The fact that the QS system is effective in the infection process has resulted in an increasing number of studies on the inhibition of the system. For this purpose, many natural and synthetic molecules have been the subject of studies and especially plants have been the focus of studies due to their rich content. In this study, one of the subjects investigated was the inhibition of violacein pigment in *C. violaceum* and as a result of the tests performed a statistically significant 32% inhibition of *C. medica* L. was observed. In the literature review, no studies on pigment inhibition in *C. violaceum* with *C. medica* species were found. Additionally in this study, *C. medica*, whose antiquorum sensing activity was investigated and was found to inhibit the production of pyocyanin pigment production in *P.aeruginosa* PAO1 strain by 20%, while it was found to have a high inhibition effect on biofilm formation with a high rate of 88%. Biofilm consists of groups of bacteria attached to surfaces and encapsulated in a hydrated polymeric matrix. *P. aeruginosa* biofilms cause persistent infections in individuals with major health problems (30). Therefore, their treatment is of great importance and alternative approaches to antibiotics are gaining attention day by day. *Campylobacter jejuni*'s antibacterial efficacy against *C. medica* L. by products (peel, seeds) was evaluated by Castillo et al. The extract decreased the production of biofilms (60–75%) and swarm motility (35–40%) (31). In our results biofilm inhibition rate was 88% and the difference between the studies is due to differences in the region where the material grows, the concentrations studied and the types of microorganism.

5. Conclusion

In recent years, the use of plant-derived extracts has received increasing attention due to concerns over possible adverse health effects caused by the use of traditional medicines. In particular, antibiotic resistance has become a global problem and new approaches to combat it have become mandatory. In this study, some of the constituents of *C. medica* L. were investigated and their effects on bacteria were also examined. In conclusion, based on the current literature review and this study, it is possible to say that *C. medica* L. can be considered as a good candidate for the treatment of various pathologies related to microbial infection and prevention of biofilm formation. However, further studies are needed to maximize the potential of *C. medica* L. on human health.

Conflicts of interest: There are no conflicts of interest among the authors.

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