



Investigation of Antibacterial and Antioxidant Activity of *Citrus medica* L Extract on Human Pathogenic Bacteria

Mohadeseh Shojaemehr¹, Mostafa Alamholo^{1*}, Jalal Soltani²

¹Department of Plant Biotechnology, Faculty of Agriculture, Bu Ali Sina University, Hamadan, Iran

²Department of Plant Protection, Faculty of Agriculture, Bu-Ali Sina University, Hamadan, Iran

***Corresponding author:**

Mostafa Aalamholo,
Department of Plant
Biotechnology, Faculty of
Agriculture, Bu Ali Sina
University, Hamadan, Iran
Tel: +989372028192
Email: mostafaalamholo@
yahoo.com

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Abstract

Background: Natural products derived from medicinal plants are a major source of drug preparation and the main basis for the development of pharmaceutical leads. We have aimed at investigating in vitro antibacterial and antioxidant activity of various extracts of *Citrus medica* L. against a number of human pathogenic bacteria.

Methods: The plant samples of *C. medica* L were collected from Ramsar province, Iran. The gram-positive bacteria *Streptococcus pyogenes*, *Bacillus subtilis*, *Bacillus cereus*, *Micrococcus luteus*, *Enterococcus faecalis*, and *Staphylococcus aureus*, as well as the gram-negative bacteria *Escherichia coli*, *Shigella boydii*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* were prepared from Bu Ali Sina University, Hamadan, Iran. Agar diffusion assay was applied, and the antioxidant properties of extracts were determined by DPPH assay. Total phenolic and flavonoid contents as well as some compounds such as alkaloids, saponin, and tannin were further analyzed.

Results: Results indicated that *C. medica* extracts possessed antibacterial activity, and that root, seed, and leaf exhibited the highest activities against human pathogens, especially *M. luteus*. Roots contained the highest total phenolics (106.1 mgGA/g), while leaves contained the highest total flavonoids (3.24 mgQ/g). Leaf methanol extract also contained alkaloids, saponins, and tannins.

Conclusions: The antibacterial activities of *C. medica* extracts could be explained by synthesizing such compounds. Moreover, seed and root extracts of *C. medica* showed strong radical scavenging activities.

Keywords: Antibacterial, *Citrus medica*, Antioxidant, Phenol, Flavonoid

Background

Medicinal plants are a rich source of lead compounds for traditional and modern medicines (1). Currently, clinical effectiveness of many antibiotics is compromised by the emergence of resistant pathogens. Therefore, there is a continuing and urgent need for the discovery of new antimicrobial sources. Herbal drugs are widely used in ethnomedicine due to their unknown bioactive compounds, low side effects, and relatively low costs (2). Accordingly, several studies have focused on medicinal plants to find more effective drugs against microbial infections (3,4). *Citrus medica* L. is a valuable medicinal plant used in Iranian ethnomedicine. It is a small plant, having short thorns and large and rectangular leaves with elliptical fruits. It is reported that *C. medica* extract plays a role in the treatment of diabetes and Alzheimer disease (5). The root extract of *C. medica* is used for its anthelmintic and antilithic properties in the treatment of urinary calculi in India (6).

It is known that plant oriented compounds such as

carotenoids, phenolics, flavonoids, and ascorbic acid eliminate free radicals and have antioxidant and anti-mutagenic properties (7). Antimicrobial activity of saponin and tannins (8), alkaloids (9), and flavonoids have been reported (10). Therefore, differences in the sensitivity of bacteria to plant extracts could be either due to the intrinsic sensitivity of microorganisms, or the nature of compounds with antimicrobial properties such as alkaloids, tannins, saponins, phenols, glycosides, and flavonoids (11,12). Moreover, several parameters affecting the plant extract efficacy are extraction method, plant genotypes (13), the moisture content of the plant, and the temperature of the extraction time (1). As a result, the extraction method, extraction time, solvent and tissue type, as well as the plant species affect the presence and the amount of desired compounds in the extracts (14).

To our knowledge, no research has been done on the antimicrobial activity of Iranian *C. medica*. Therefore, here we aimed at investigating antibacterial and antioxidant activities of various extracts of *C. medica*

against some human pathogenic bacteria in vitro. We further investigated the presence of flavonoids, saponins, and tannins in methanol extracts of *C. medica* in order to find a possible mechanism for such an effect.

Materials and Methods

Chemicals

Nutrient broth (NB), Mueller-Hinton Agar culture media, DPPH (2,2-diphenyl-1-picrylhydrazyl), Quercetin and Gallic acid were purchased from Merck Co. (Darmstadt, Germany). Ciprofloxacin and Gentamicin discs were prepared from Paten Tab Co. (Tehran, Iran).

Preparation of the Plant Extracts

The plant samples of *C. medica* were collected from Mazandaran province, north of Iran. The samples were immediately transferred to the laboratory and dried at room temperature in the shade and far from direct sunlight. Dried samples were broken into small pieces. Ethanol (96%), methanol (80%), and distilled water extracts were obtained by soxhlet extractor (15). To this end, 30 g of dried powder was separately added to 350 mL of each solvent used. After 4 hours, the extracts were filtered through filter paper and centrifuged at 10 000 rpm for 8 minutes (16). The extract solvent was evaporated by a rotary evaporator and was transferred to an oven at 37°C for complete drying. The residue was stored in the dark at -22°C.

Bacterial Strains and Culture Conditions

All bacteria were obtained from Hamadan University of Medical Sciences, Iran. Antibacterial activity of the extracts were tested in vitro against Gram-positive bacteria *Streptococcus pyogenes* (PTCC-1447), *Bacillus subtilis* (PTCC-1156), *Bacillus cereus* (PTCC-1247), *Micrococcus luteus* (ATCC 10987), *Staphylococcus aureus* (PTCC-1189), and *Enterococcus faecalis* (PTCC-1195) and Gram-negative bacteria *Escherichia coli* (ATCC-25922), *Shigella boydii* (PTCC1744), *Salmonella typhi* (PTCC-1609), *Pseudomonas aeruginosa* (PTCC-1181) *Enterobacter aerogenes* (PTCC-1221), and *Klebsiella pneumoniae* (PTCC-1129). For preparation of fresh bacterial cultures, a bacterial colony was transferred to MHA medium and incubated at 37°C for 24 hours. Then, a loop of bacterial colony was transferred to 1 mL NB medium and incubated at 37°C for 18 hours. The turbidity of the suspension was adjusted to 0.5 McFarland standard (1.5×10^8 CFU).

Agar Well Diffusion Assay for Assessing the Antibacterial Activity

Agar well diffusion assay was used to determine the antibacterial activity of plant extracts (17). The concentrations of 400 mg/mL (root, seed, or leaf extract) of ethanol (96%), methanol (80%) and distilled

water were prepared. A volume of 200 mL of bacterial suspension (1.5×10^8 CFU) was poured onto MHA medium and uniformly spread with a swab. Then, 5 mm diameter wells were created in Petri plates, and 50 µL of the extract was poured into each well. Petri plates were incubated at 37°C for 24 hours (18). Gentamicin (10 µg) and ciprofloxacin (0.005 µg) were used as positive controls (19). The inhibitory zone formed around each well was measured (cm). The results from three replicates of the experiment were statistically analyzed.

Determination of MIC and MBC

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanol, methanol and distilled water extracts were determined by serial dilution method (20). Dilution series of 400, 200, 100, 50, 25, 12.5, and 6.25 mg/mL were prepared for MIC. A volume of 15 µL of each bacterial suspension (0.5 McFarland) was added to each test tube. The positive (300 µL of the extract with 285 µL of culture medium) and negative (285 µL of the extract with 15 µL of bacteria) controls were considered. The tubes were incubated at 37°C for 24 hours. The lowest dilution of the extract with no growth of bacteria was considered as MIC. To measure MBC, 5 µL of the tubes with no bacterial growth was added on MHA culture medium. Plates were incubated at 37°C for 24 hours. The minimum concentration with no bacterial growth on the plates was considered as MBC.

Determination of Total Phenolic Content

Total phenolics content was estimated according to the Folin-Ciocalteu method (21). Accordingly, 100 mg of extract was dissolved in 1 mL of methanol. Then, 2 mL of sodium carbonate (Na_2CO_3) was poured into a tube and vortexed. Afterwards, 2.5 mL of 10% Folin solution was added to the solution and remixed. After 15 minutes at 25°C in darkness, the absorbance of samples was measured at 765 nm using a spectrophotometer and determined as mg of gallic acid per gram of dry extract weight (mg GA/g).

Determination of Total Flavonoids Content

Total flavonoids content was determined by aluminum chloride method (22). Accordingly, 100 µL of 10% aluminum chloride and 100 µL of potassium acetate were poured into a tube and mixed. Then, 2.8 mL of distilled water and 0.5 mL of extract solution were added. After 30 minutes at room temperature, the absorption of samples was measured at 415 nm using a spectrophotometer and determined as mg of quercetin per gram of dry extract weight (mg Q/g).

Determination of DPPH for Free Radical Scavenging Activity

Free radical activity was investigated according to

Stojičević et al (23). Different concentrations (0.2, 0.4, 0.6, 0.8, and 1 mg/mL) of methanol extracts of root, seed, and leaf were prepared and ascorbic acid was used as the standard. The samples were placed in darkness for 30 minutes, and then, solvent absorption was recorded with a spectrophotometer at 517 nm. Methanol (99%) was used as the blank. The free radical scavenging activity (%) was calculated by the following formula:

$$\text{RSAC (\%)} = 100(1 - (A_s - A_b)/A_c)$$

Where A_s indicated sample; A_b indicated blank; and A_c indicated control.

Identification of Tannin, Saponin, and Alkaloids

To investigate the presence of alkaloids, 0.5 g of methanol extract was dissolved in 5 mL of 1% HCl and kept for 5 minutes in a warm distilled water bath. Then, the solution was passed through a filter paper, and a few drops of Mayer's reagent were added to it (24). The sedimentation or turbidity indicated the presence of alkaloids (25). To track tannin, 0.5 g of methanol extract was dissolved in 5 mL of distilled water and the solution was passed through filter paper. Then, a few drops of FeCl₃ chloride (10%) were added to it. The appearance of the black-green color indicated the presence of tannin (26).

To track saponin, 20 mL of distilled water was added to 0.25 g methanol extract and boiled. The solution was passed through a filter paper, and 5 mL of it was mixed with 20 mL of distilled water and shaken. The formation of the stable foam indicated the presence of saponin (27).

Statistical Analysis

The experiments were performed in a completely randomized design with factorial test. The average comparisons were done using Duncan test at $P < 0.05$

using SPSS software version 16.0.

Results

Antibacterial Activity

The inhibitory effects of different alcoholic and aqueous extracts of root, seed, and leaf of *Citrus medica* were evaluated against human pathogenic bacteria (Table 1). Negative control (50 µL of used solvents) and positive controls (gentamicin and ciprofloxacin) were included. After incubation, diameters of zone of inhibition around the wells were measured. Data indicated that the methanol extracts of leaves showed a better inhibitory effect on *B. cereus*, *S. aureus*, *M. luteus*, and *E. coli*. Inhibitory activity of leaf methanol extract on *B. cereus*, *E. coli*, and *E. aerogenes* was more potent than gentamicin. Furthermore, methanol extracts of roots showed the highest inhibitory activity against *M. luteus*. The inhibitory activity of root methanol extract on *M. luteus* and *E. aerogenes* and that of root ethanol extract on *E. aerogenes* was more potent than gentamicin. The methanol extract of seeds showed the highest inhibitory activity against *M. luteus*; however, *S. pyogenes*, *S. boydii*, and *P. aeruginosa* showed resistance against aqueous extracts. Additionally, *Shigella boydii* showed resistance against the methanol, ethanol, and aqueous extracts of seeds. In total, seed extracts showed less inhibitory activity than the leaves and roots extracts (Table 1).

The MIC of the methanol extract of leaves against *E. coli* was 6.25 mg/mL. MIC of root, leaf, and seed methanol extract against *B. subtilis*, *S. aureus*, and *S. typhi* were 12.5 mg/mL. However, no inhibitory effect was observed against *S. pyogenes* and *S. boydii*; therefore, these bacteria were deleted from Table 2. The leaf aqueous extract

Table 1. Antibacterial Activity of Methanol, Ethanol, and Aqueous Extracts of Root, Leaf and Seed of *Citrus medica* in Comparison to Gentamicin and Ciprofloxacin

Bacteria	Leaf			Root			Seed			Gentamicin	Ciprofloxacin
	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous		
<i>B. subtilis</i>	10±0.57	11.33±0.33	9.33±0.33	13.66±0.33	12±1.54	11.67±0.88	10.67±0.33	17±0.57	11.33±0.33	29±0.57	29.5±0.33
<i>B. cereus</i>	20±0.57	13.33±0.33	6.33±0.66	11.33±0.66	12.33±1.33	11±0.57	15.67±0.33	14.67±0.66	10.33±0.33	19.66±0.33	28.5±0.66
<i>S. aureus</i>	20±0.57	16.33±0.67	12.67±0.33	13.33±0.66	12.67±0.33	12.67±0.33	8.67±0.33	11.67±0.33	9.33±0.33	20±1	28.5±0.66
<i>S. pyogenes</i>	8.33±0.33	10.33±0.33	0±0	6.67±0.33	7±0.57	6.33±0.33	7.67±0.33	8.67±0.33	0±0	20±0.57	31.5±0.33
<i>M. luteus</i>	21±0.57	14.67±0.33	12.67±0.33	25±1.73	14.67±0.33	6.67±0.66	20.67±0.33	18.33±0.88	11.33±0.33	22±0.33	30±1
<i>E. faecalis</i>	19±0.33	19±0.58	15±0.88	18±0.66	16±0	13±0.33	17±0.33	15±0.88	13±0.88	23±0.88	25±0.33
<i>S. typhi</i>	12.67±0.88	13.67±0.33	12.33±0.33	18±0.57	10.33±0.33	10.33±0.33	19.67±1.2	13.33±0.33	12.67±0.66	29.5±1	33±0.57
<i>S. boydii</i>	9.33±0.33	10.33±0.66	9.67±0.33	13±0.57	11.67±0.33	9.33±0.66	0±0	0±0	0±0	19±0.57	37.5±0.66
<i>P. aeruginosa</i>	9±0.57	9.33±0.33	8±0	18.33±0.66	14.33±0.33	8.33±0.88	14.33±0.33	0±0	0±0	20±0.33	24.5±0.66
<i>E. coli</i>	21±0.57	17.67±0.33	14.67±0.67	12.67±0.33	14±0.57	8.33±0.33	7.67±0.33	11±0.57	9.33±0.33	19.5±1	24.5±0.57
<i>E. aerogenes</i>	17±1	13±0.57	9.33±0.33	17.67±0.33	19±0.57	12.57±0.33	9.67±0.33	11.67±0.33	8±1	11±0.33	28±0.33
<i>K. pneumoniae</i>	18±0.33	19.5±0.57	14±0	21±0.88	19±0.88	15.66±1.2	12±0	11.5±0.33	10±0	20±0.33	22±0.88

Table 2. MIC and MBC (mg/mL) of Different Extracts of *Citrus medica* Against Human Pathogenic Bacteria

Organ	Con		Bacteria									
			<i>B. subtilis</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>M. luteus</i>	<i>E. aerogenes</i>	<i>S. typhi</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>K. pneumoniae</i>
Leaf	M	MIC	50	25	12.5	50	50	100	-	6.25	100	200
		MBC	100	50	25	-	-	-	-	25	200	-
	E	MIC	50	50	50	50	25	50	-	25	50	100
		MBC	100	-	100	-	50	100	-	50	100	200
	A	MIC	-	-	100	100	-	100	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-	-
Seed	M	MIC	50	50	50	25	-	12.5	50	-	500	100
		MBC	100	25	-	50	-	50	100	-	200	100
	E	MIC	50	50	25	50	50	50	-	25	200	100
		MBC	-	-	50	100	-	-	-	50	200	200
	A	MIC	100	100	-	100	-	50	-	-	-	-
		MBC	-	50	-	-	-	-	-	-	-	-
Root	M	MIC	12.5	-	50	25	25	50	50	25	50	25
		MBC	50	-	-	50	-	100	100	50	50	100
	E	MIC	50	100	50	50	50	100	100	50	100	100
		MBC	100	-	50	-	50	-	100	100	200	100
	A	MIC	50	100	100	100	50	200	-	200	-	-
		MBC	100	100	200	200	50	200	-	200	-	-

Con: concentration (mg mL⁻¹), M: methanol, E: ethanol, A: aqueous, -: lack of growth.

did not show inhibitory activity on all tested bacteria. According to the results, methanol extract showed more inhibitory activity compared to ethanol and aqueous extracts. In addition, gram-positive bacteria such as *B. subtilis*, *B. cereus*, and *M. luteus* were more sensitive than gram-negative bacteria. The tested bacteria showed better sensitivity against root (Table 2). The aqueous extract did not show any effect on *E. faecalis* and *K. pneumoniae*.

Antioxidant Activity of *Citrus medica* Extracts

As seen in Table 3, the amount of DPPH free radicals inhibition was increased by increasing the concentration of plant extracts. A significant difference was observed between the IC₅₀ values of methanol extracts and ascorbic acid as the control (Table 3). Seed and root methanol extracts of *C. medica* showed strong radical scavenging activities.

Total Phenolic and Flavonoid Content

The results of total phenolic and flavonoid content of root, seed and leaf methanol extracts of *C. medica* have been shown in Table 4. Total phenolic contents of root, seed, and leaf were determined as 106.1, 103.8, and 102.7 mgGA/g, respectively. The contents of flavonoids were determined as 3.24, 3.02, and 3.96 mgQ/g, respectively.

The Presence of Alkaloids, Tannins, and Saponins

The methanol extract, which showed the highest antimicrobial activity, was used to study the presence and absence of alkaloids, saponins, and tannins. The results of the presence and absence of alkaloids, saponins, and tannins have been shown in Table 5. Alkaloids, saponins, and tannins were observed in the methanol extract of leaf, while the methanol extracts of root and seed showed only the presence of alkaloids.

Discussion

According to the obtained results, the methanol extract showed better inhibitory activity on tested bacteria compared to ethanol and aqueous extracts. The highest inhibitory zone diameter was observed against *M. luteus* against the root methanol extract. The MIC and MBC of root, seed, and leaf tissues were not reported against *S. pyogenes* and *S. boydii*. Secondary metabolites such as phenol and flavonoids have a strong potential for clearing free radicals that are present in all parts of plants such as leaves, fruits, seeds, roots, and peels (28). The emergence of new bacterial strains with multiple resistances, unavailability, and high costs of chemical drugs has led to an increase in mortality in the world (28,29). Therefore, it is necessary to find compounds with antimicrobial

Table 3. Antioxidant Activity (IC₅₀: mg mL⁻¹) of Different Extracts of *Citrus medica* and Inhibition Percentage of the DPPH

Tissue/ Chemical	Inhibition Percentage of DPPH of the Different Concentration (mg/mL)					IC ₅₀
	0.2	0.4	0.6	0.8	1	
Root	74.68	83.03	87.4	91.53	93.3	0.133 ^b
Seed	68.37	76.43	84.72	88.33	91.73	0.146 ^a
Leaf	89.61	95.51	97.36	96.22	96.47	0.111 ^c
Ascorbic acid	91.3	92.43	97.41	98.56	99.67	0.109 ^c

Note. Same letters are not significantly different at $P < 0.05$.

Table 4. Total Phenolics and Flavonoids Contents of Methanol Extracts of Root, Seed, and Leaf of *Citrus medica*

Organ	Root	Seed	Leaf
Phenol (mgGA/g)	106.1 ^a	103.8 ^b	102.7 ^b
Flavonoid (mgQ/g)	3.24 ^b	3.02 ^b	3.96 ^a

Note. Same letters are not significantly different at $P < 0.05$

Table 5. Alkaloids, Saponins and Tannins Contents in the Methanol Extract of *C. medica* Tissues

Tissue	<i>C. medica</i>		
	Alkaloid	Saponin	Tannin
Root	+	+	+
Leaf	+	-	-
Seed	+	-	-

+: Presence -: Absence

properties from medicinal plant sources (14). The ethanol and methanol (polar solvents) can better dissolve the polar compositions in the plant and have antibacterial effects on pathogenic microorganisms (30).

Sah et al (13) tested antimicrobial properties of root and leaf ethanol extract of *C. medica* against human pathogenic bacteria. The MIC of the root was 0.5 mg/mL against *B. subtilis*, which is not in line with our results. Kabra et al (3) investigated the antimicrobial effect of ethanol extract of *C. medica* peel on human pathogenic bacteria and reported that *S. aureus* and *P. aeruginosa* had more sensitivity against the tested extract. In our research, the seed ethanol extract did not show any inhibitory effect against *P. aeruginosa*, which is contrary to the results of the mentioned group. These differences probably could be due to compound extraction methods, plant genotype, type of organ and the time and stage of plant growth.

Menichini et al (31) reported that IC₅₀ of hexane extract of *C. medica* skin was 0.471 mg/mL, which was almost similar to our results. Reddy et al (32) reported the highest percentage of free radical scavenging for the leaf methanol and hydro methanol extracts of *C. aurantifolia*, equal to 93.53. Ghasemi et al (33) reported

that IC₅₀ values of methanol extract of 13 Iranian *Citrus* species ranged from 0.6 to 2.9 mg/mL. Differences in the chemical composition and secondary metabolites with antimicrobial properties such as phenol, ascorbic acid, and carotenoids, as well as differences in species can affect the level of antioxidant changes (2).

Choudhury et al (12) related the antimicrobial properties of plant extracts to the compounds such as phenolic, flavones, tannins, terpenoids, alkaloids, flavonoids, and saponin. Tannin showed a good in vitro antimicrobial activity (34). Some flavonoids with anticancer properties including hesperidin, narirutin, naringin, and eriocitrin were reported (35). The antimicrobial activity of saponins has been shown on some microorganisms (36). Gorinstein et al (37) determined the total phenolic content of lemon and orange peel as 1.9 and 1.8 (mgGA/g). The total flavonoid contents of ethanol extract of *C. sinensis*, *C. maxima*, and *C. reticula* were estimated as 0.13, 0.20, and 0.14 (mgQ/g), respectively (38), which are different from our results. These differences could be probably due to differences in species type, tissue type, and extraction method. Ghasemi et al (33) analyzed 13 species of Iranian *Citrus* and reported that total phenolic content of the *Citrus* spp. samples varied from 66.5 to 396.8 mgGA/g and flavonoid content varied from 0.3 to 31.1 mgQ/g. These results are approximately similar to the results of the present study.

Karou et al (39) reported the presence of alkaloids and the absence of saponins and tannins in ethanolic extract of *C. medica* by phytochemical methods. Bairagi et al (40) showed the presence of alkaloid and the absence of saponin and tannin in the leaf extract of *C. medica*. Wu et al (41) reported the presence of alkaloid in root acetone extract of *C. grandis*, and the presence of tannin, alkaloids, and saponin was reported in leaf diethyl ether extract of *C. grandis* (42). Pandey et al (16) reported the presence of tannin and the absence of saponin in the seed methanol extract of *C. limon*. Pathan et al (43) reported the presence of tannin in leaf and the presence of alkaloids in the leaf and root hydroalcoholic extract of *C. aurantium*. Reddy et al (32) by phytochemical analysis showed the presence of alkaloid, saponin, and tannin in the leaf methanol extract of *C. aurantium*.

Conclusions

Our results showed that the alcoholic extracts of *C. medica* contain antibacterial compounds. These results could be useful in the identification of active compounds that can be formulated into antibacterial herbal drugs. This study suggests that *C. medica* may be used to discover natural bioactive products which might lead to the development of new drugs with antibacterial and antioxidant properties in the field of medicine.

Conflict of Interest Disclosures

None.

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Ethical Statement

Not applicable.

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