Research in Molecular Medicine

Antimicrobial Activity of Ethanolic and Methanolic Extracts of *Urtica dioica*, *Mentha longifolia*, and *Bacteriocin* Produced by *Lactobacillus casei* Against Antibiotic-resistant Bacteria

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ABSTRACT

Background: The increasing resistance of human microbial pathogens to the available antibacterial compounds is a significant threat, resulting in the search for new antibiotic resources such as plants and probiotics. Therefore, this study aimed to evaluate the antibacterial effect of ethanolic and methanolic extracts of *Urtica dioica, Mentha longifolia*, and bacteriocin purified from a probiotic bacteria using the standard disk diffusion method against some pathogenic strains.

Materials and Methods: Ethanolic/methanolic extract of *U. dioica, M. longifolia*, and bacteriocin from probiotic bacteria were prepared by the standard methods. The effect of different concentrations of the extracts on some antibiotic-resistant bacteria was evaluated using the standard disk diffusion method by measuring the diameter of the growth inhibition zone.

Results: The disk diffusion test showed that the bacteriocin *Lactobacillus casei* had more growth inhibitory effects on the tested bacterial strains than the methanolic and ethanolic extracts of *U. dioica* and *M. longifolia*. Bacteriocin extract of *L. casei* exhibited significant antibacterial activity at the concentrations of 12 and 18 mg/mL ($P \leq 0.05$) against antibiotic-resistant bacteria, while a 12 mm zone of inhibition was observed in the concentration of 1.5 mg/mL against *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*).

Conclusion: According to the agar well diffusion method results, the bacteriocin producing *L. casei* has an extensive range of antibacterial spectrum against resistant bacteria. It can be used as an alternative to antimicrobia agents for the treatment of infections caused by resistant bacteria. It is suggested that in future research, the cytotoxicity of the extracts be evaluated in vitro/in vivo studies..

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Introduction

Although pharmacological activities have introduced and produced several new antibiotics in the last couple of decades, resistance to this antibiotic by human pathogenic bacteria has increased [1-3]. Overall, pathogenic bacteria have the genetic capability to acquire and transmit resistance to these antibiotics, which are used for therapeutic purposes [4, 5]. Nowadays, medicinal herbal plants and probiotic production of bacteria have many applications in human infections [6]. People in Asia, Africa, and Latin America use plants as a form of medication which accounts for approximately 80% of the world use old-fashioned health therapies and they are described to have the least side effects [7]. Two of these herbal plants, i.e. *U. dioica* and *M. longifolia* are often used therapeutically as a traditional treatment for different diseases. *U. dioica* with the Latin name nettle is a member of the Urticaceae class and has many significant purposes in ancient therapy due to it having various remediable effects [8, 9].

M. longifolia (Family: Lamiaceae, Class: Magnoliopsida) is an important herbal plant in the traditional system of Ayurveda used for the treatment of several chronic diseases and is widely scattered in India, Pakistan, Sri Lanka, Iraq, Iran, Turkey, Egypt, Greece, China, and Mediterranean areas [10, 11]. Several studies have reported that *M. longifolia* and *U. dioica* have an excellent antimicrobial effect against different pathogenic bacteria [11-16]. Besides, probiotic Lactic Acid Bacteria (LAB) such as *L. casei, Lactobacillus gasseri, Lactobacillus acidophilus, Lactobacillus johnsonii, Lactobacillus rhamnosus, and Lactobacillus reuteri* are the most common strains of microbes used as probiotics [17]. These bacteria can produce different antimicrobial components, including organic acids, low-molecular-weight antimicrobial substances, and bacteriocins [18]. The probiotic bacteria such as L. *acidophilus* and *Bifidobacterium* spp. are increasingly used in pharmaceutical applications and they have excellent therapeutic benefits [19]. Amin et al. indicated that the isolation of 60 LAB strains from vegetables in MRS broth medium (Man-Rogosa-Sharpe) showed significant antibacterial activity against pathogenic bacteria such as *Escherichia coli, Shigella dysenteriae, Salmonella typhi, Staphylococcus aureus,* and *Bacillus anthracis* [20]. It is essential to focus on the substitution sources of the antimicrobial agents as the pathogenic microorganism is acquisition resistance against usual antibiotics [21]. Medicinal plants such as *Ocimum gratissimum, Vernonia amygdalina,* and *Aframomum melegueta* have been ascertained to provide various culinary and medicinal properties. These medicinal properties exert bacteriostatic and bactericidal effects on some bacteria [21, 22]. This in vitro study aimed to determine the antimicrobial effect of *U. dioica* and *M. longifolia*, and antimicrobial properties of selected probiotic bacteria (*L. casei*).

Materials and Methods

Plant Collection

U. dioica was collected from Lahijan City and *M. longifolia* was collected from the Zabol City. Samples were collected from May to June of 2015.

Extract Preparation

The aerial fragments of *U. dioica* and *M. longifolia* were shade-dried at room temperature for 15 days and then ground to a fine powder using an electronic blender. The extract was obtained from 1 g of the powder using 10 mL of alcohol (ethanol or methanol) (Merck, Germany) and distilled water solu-tion, centrifugation at 3000 rpm for 15 minutes, and collecting the supernatant. This procedure was repeated three times and the solvents were evaporated by placing the yielded materials at room temperature for 7 days [23, 24].

Bacterial Strains

In this experimental study, the following American Type Culture Collection (ATCC) strains of bacteria were used: methicillin-resistant *Staphylococcus aureus* ATCC 43300, vancomycin-resistant *Enterococcus faecalis* ATCC 51299, carbapenem-resistant *Pseudomonas aeruginosa* ATCC 2113, carbapenem-resistant *Acinetobacter baumannii* ATCC 1605, carbapenem-resistant *Klebsiella pneumoniae* ATCC 1705, and clinical isolates of *S. Typhimurium* were obtained from the Medical Sciences Culture Collection in Tehran, Iran. By conventional microbiological and biochemical tests, the tested strains were identified. Antimicrobial susceptibility testing (AST) of the tested strains was re-checked using the Kirby-Bauer disk diffusion method as described by CLSI 2018 [25]. Also, *L. casei* ATCC 39392 as a probiotic strain was obtained from the Persian variety of the culture collection (Tehran, Iran).

Production of crude bacteriocin

To extract the bacteriocin, *L. casei* was cultured in 250 mL of the MRS broth for 72 h at 30°C anaerobically. The supernatant was obtained through centrifugation at 10000 rpm for 10 min and was adjusted to pH 7.0. Then,

the supernatant was precipitated with 40% ammonium sulfate and the mixed reaction was rotated for 1 h and centrifuged at 10000 rpm for 15 minutes. The precipitates were dialyzed and re-suspended in 10 mL of 0.05 M potassium phosphate buffer with pH 7.0 [26].

Antimicrobial Susceptibility Testing

The antibacterial activities of the extracts and bacteriocin produced by *L. casei* were checked using the agar well diffusion method [27-29]. The test strains were grown on blood agar medium plates for 24 h at 37°C. Single colonies from the strains were suspended in sterile Mueller– Hinton broth (Merck company, Darmstadt, Germany), and the turbidity was adjusted against 0.5 McFarland standard concentration (1.5 × 108 CFU/mL). Then, the prepared suspension was inoculated on Mueller Hinton agar medium (Merck, Darmstadt, Germany). Blank disks (6.4 mm) were saturated by different doses of the extract (1.5, 3, 6, 12, and 18 mg) were placed on lawn cultures after solvent evaporation. Then the plates were incubated for 24 h at 37°C [30, 31]. Each experiment was conducted three times to observe an effective outcome. Also, filter paper disks inoculated with sterile saline and fosfomycin were used as negative and positive control, respectively. The inhibition zone around each disk was measured in mm using a ruler [32].

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS 20; SPSS Inc., Chicago, IL, USA) was used to study the differences between the means in the study parameters. The results were considered significant when P values were less than 0.05.

Results

The standard disk diffusion assay was done to determine the presence of antibacterial activities. The antimicrobial activity of ethanolic and methanolic extracts of *U. dioica* appeared as a growth inhibition factor for MRSA ATCC 43300 and VREF ATCC 51299; however, these two extracts of the *U. dioica* have minimal inhibition effect on CRPA ATCC 2113, CRAB ATCC 1605, and CRKP ATCC 1705. The results of the antimicrobial susceptibility of *U. dioica* are presented in Table 1. The highest activity (inhibition zone diameter about 18 mm) was demonstrated by the methanolic extract of *U. dioica* against VREF ATCC 51299 while the lowest activity (inhibition zone diameter about 4 mm) was revealed by the ethanolic extract against CRAB ATCC 1605 and CRKP ATCC 1705. The antibacterial susceptibility of ethanolic and methanolic extracts of *M. longifolia* appeared to inhibit the growth of CRPA ATCC 2113, CRAB ATCC 1605, and CRKP ATCC 1705. However, these two extracts of *M. longifolia* has a minimal inhibition effect on MRSA ATCC 43300 and VREF ATCC 51299.

The results of the antimicrobial activity of *M. longifolia* are presented in Table 2. The highest activity (inhibition zone diameter about 19 mm) was demonstrated by the methanolic extract of *M. longifolia* against CRPA ATCC 2113 while the lowest activity (inhibition zone diameter about 7 mm) was demonstrated by the ethanolic

Table 1. Antibacterial activity of the ethanolic and methanolic extracts of U. dioica against pathogenic bacteria

Organisms	Mean Zones of Inhibition (mm)									
	Concentration of Extract (mg/mL)									
	Ethanolic Extract					Methanolic Extract				
	18	12	6	3	1.5	18	12	6	3	1.5
MRSA ATCC 43300	14	12	10	8	7	16	15	12	11	9
VREF ATCC 51299	17	15	13	12	11	18	15	12	10	8
CRPA ATCC 2113	11	9	8	7	6	13	11	10	9	8
CRAB ATCC 1605	12	8	7	4	No effect	11	9	8	No effect	No effect
CRKP ATCC 1705	11	9	6	4	No effect	12	10	9	No effect	No effect
S. Typhimurium	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect

Methicillin-resistant *Staphylococcus aureus* (MRSA); Vancomycin-resistant *Enterococcus faecium* (VREF); Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA); Carbapenem-resistant *Acinetobacter baumannii* (CRAB); Carbapenem-resistant *Klebsiella pneumoniae* (CRKP)

Table 2. Antibacterial susceptibility of the ethanolic and methanolic extracts of M. longifolia against pathogenic bacteria

Organisms	Mean Zones of Inhibition (mm)									
	Concentration of Extract (mg/mL)									
	Ethanolic Extract					Methanolic Extract				
	18	12	6	3	1.5	18	12	6	3	1.5
MRSA ATCC 43300	8	7	No effect	No effect	No effect	9	6	No effect	No effect	No effect
VREF ATCC 51299	11	7	No effect	No effect	No effect	10	7	No effect	No effect	No effect
CRPA ATCC 2113	17	14	12	11	9	19	17	16	15	12
CRAB ATCC 1605	16	14	11	9	7	18	17	14	12	11
CRKP ATCC 1705	18	16	14	13	11	16	15	13	10	9
S. Typhimurium	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect

Methicillin-resistant *Staphylococcus aureus* (MRSA); Vancomycin-resistant *Enterococcus faecium* (VREF); Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA); Carbapenem-resistant *Acinetobacter baumannii* (CRAB); Carbapenem-resistant *Klebsiella pneumoniae* (CRKP)

Table 3. Antibacterial activity of *L. casei* purified bacteriocin against pathogenic bacteria

Organisms	Mean Zones of Inhibition (mm)				
	Concentration of purified bacteriocin (mg/mL)				
	1.5	3	6	12	18
MRSA ATCC 43300	21	20	19	18	17
VREF ATCC 51299	24	20	16	15	14
CRPA ATCC 2113	22	20	18	16	14
CRAB ATCC 1605	18	17	16	15	13
CRKP ATCC 1705	13	12	11	9	7
S. Typhimurium	12	11	9	8	6

Methicillin-resistant *Staphylococcus aureus* (MRSA); Vancomycin-resistant *Enterococcus faecium* (VREF); Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA); Carbapenem-resistant *Acinetobacter baumannii* (CRAB); Carbapenem-resistant *Klebsiella pneumoniae* (CRKP)

extract against CRAB ATCC 1605. On the other hand, the bacteriocin extracts of *L. casei* were more active against 4 strains of MRSA ATCC 43300, VREF ATCC 51299, CRPA ATCC 2113, and CRAB ATCC 1605. The highest activity (inhibition zone diameter about 19 mm) was demonstrated by the bacteriocin extracts of *L. casei* against VREF ATCC 51299, while the lowest activity (inhibition zone diameter about 6 mm) was demonstrated by the bacteriocin extracts of *L. casei* against *S. Typhimurium* (Table 3).

Discussion

Although the treatment of microbial infections with antibiotics seems harmless and effective, it has various problems. It has become evident that antibacterial resistance has increased and thus the development of new antibacterial agents is undoubtedly required [33]. In the current study, several resistant bacterial strains were selected to examine the antimicrobial effects of ethanolic and methanolic extracts of the *U. dioica* and *M. longifolia*. In addition to this, the antibacterial activity of bacteriocin *L. casei* was evaluated against resistant bacterial strains. Several scientists have analyzed and described

the antimicrobial activities of various plants [34, 35]. In ancient treatment, *U. dioica* has been identified for its several therapeutic properties such as cardiovascular [36], anti-rheumatic, anti-inflammatory [24, 37], acute diuretic effects, hypotensive, and natriuretic [8].

Gulcin et al. reported that the antimicrobial activity of the water extract of *U. dioica* against *P. aeruginosa* ATCC 9027, *E. coli* ATCC 9837, *S. aureus* ATCC 6538, and *S. pneumoniae* ATCC 49619 [9]. Our results were similar to the findings of Gulcin et al. [9] and all bacteria have been inhibited by the *U. dioica* plant extract. Ibrahim et al. reported that the botanical extract of the *U. dioica* significantly inhibited the cell growth of MRSA [38]. Heidary et al. reported that the extracts of the *U. dioica* had high [34, 35] antibacterial effects against β-lactamase producing *P. aeruginosa* [39].

According to our results, butanol ethanolic and methanolic extracts of *U. dioica* can inhibit MRSA strains. Also, ethanolic and methanolic extracts of the *U. dioica* could inhibit some resistant strains. This antibacterial activity may be related to alkaloid, tannins, terpenes glycosides and phenol components that were present in nettle [16]. *S. Typhimurium* was resistant to ethanolic and methanolic extracts of the *U. dioica*. *M. longifolia* is an aromatic stimulant used for alleviating nausea, vomiting, and headaches. In the study of Bakht, all extracts from *M. longifolia* displayed different ranges of antibacterial activities [40].

Hajlaoui et al. reported that the essential oil of *M. longifolia* ssp. longifolia has great antimicrobial activity potential against *S. epidermidis* CIP 106510, *S. aureus* ATCC 25923, *Micrococcus luteus* NCIMB 8166, *E. coli* ATCC 35218, *Listeria monocytogenes* ATCC 19115, *E. faecalis* ATCC 29212, P. aeruginosa ATCC 27853 and *S. Typhimurium* LT2 DT104 [41]. Gulluce et al. exhibited a strong antibacterial activity of the *M. longifolia* L. ssp. Longifolia oil against various microorganisms [11]. Our outcomes determine that ethanolic and methanolic extracts have a stronger and wider spectrum of antibacterial activity against resistant bacteria. These results were in accordance with those reported by Gulluce et al. [11]. *S. Typhimurium* was resistant to both ethanolic and methanolic extracts of *M. longifolia*. Furthermore, *L. casei* produced bacteriocin that was active against the resistant strains used in the current study.

Prema et al. reported that the bacteriocin-producing strain, *L. plantarum* displayed that the inhibitory zone ranged from 16 mm to 24 mm against *E. coli*, *Salmonella typhi, Vibrio cholerae, and S. dysenteriae* [42]. Venkatesan et al. isolated probiotic organisms, *Bifidobacterium* spp. and *Lactobacillus* spp. from soil and curd. Then, probiotic *Bifidobacterium* spp. displayed high inhibitory effect against *Salmonella* spp. [43]. In the present study, the maximum zone of inhibition (24 mm) was recorded against VREF ATCC 51299 as an indicator strain. Followed by this, 22 mm, 21 mm, and 18 mm were recorded against CRPA ATCC 2113, MRSA ATCC 43300, and CRAB ATCC 1605, respectively. In contrast, ethanolic and methanolic extracts *M. longifolia* and *U. dioica*, the bacteriocin of *L. casei* could inhibit the growth of *S. Typhimurium*. In total, the bacteriocin of *L. casei* is more effective than the alcoholic extracts of *M. longifolia* and *U. dioica* against 6 strains.

Badirzadeh reported that *U. dioica* extracts present promising antiparasitic activity and can be considered as an effective and harmless herbal material [44]. Sura et al. [45] investigated the antimicrobial activities of aquatic extracts of *U. dioica* by disk diffusionmethod. Their results showed that all the extracts can be used more safely in the treatment of digestive diseases. The present study had some limitations, the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) tests was not performed to all the tested strains in line with the agar disk-diffusion method. Also, our research only focused on references strains, and antimicrobial activities of these extracts on multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria recovered from the patients was not evaluated. In order to achieve better results, MTT assay to evaluate the cytotoxic potential of the extracts is also necessary.

The study assessed the effectiveness and antimicrobial influence of the selected probiotic strains and ethanolic and methanolic extracts of *U. dioica*, *M. longifolia* extract on antibiotic-resistant bacteria. The outcomes of this work can run some elucidations on the traditional application of effective substances of the plants tested to combat infections caused by bacteria, particularly those caused by resistant pathogens. However, more in vitro as well as in vivo studies should be carried out to gain further insight on the effect and doses of these compounds required to effectively manage bacterial resistance. As displayed through the data, the bacteriocin producing *L. casei* has a wide range of the antibacterial spectrum against resistant bacteria.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Supervision: Abazar Pournajaf, Mehrdad Gholami; Data collection: Masoumeh Kiani and Mohsen Karami; Data analysis: Mojtaba Taghizadeh Armaki and Thelma Zareh; Writing – original draft: Mehrdad Gholami and Abazar Pournajaf.

Conflict of interest

The authors declared no competing interests

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