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Determination of The Antibacterial Activities of Stinging *Nettle (*Urtica Dioica*)* Ethanol Extract at Different Bacterial Concentrations

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Abstract
The study evaluated the antibacterial effect of stinging nettle extract by the liquid microdilution method to obtain quantitative results. Unlike other studies in the literature, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values showing bacteriostatic and bactericidal effects were investigated for four different bacterial concentrations. Six pathogen strains were studied, including *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 45615, *Klebsiella pneumoniae* ATCC 70063, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and different levels of antimicrobial effects were determined. While the extract does not affect the *Klebsiella pneumoniae* ATCC 70063 strain at any level, *Pseudomonas aeruginosa* ATCC 27853 in strains with MIC values $1.5x10^5$ and $1.5x10^4$ cfu/mL were found at concentrations, but no effect was observed at the MBC level. While MIC and MBC values were found at the concentrations of *Staphylococcus aureus* ATCC 29213 except for the $1.5x10^7$ concentration, the remaining standard strains had different MIC and MBC values at all concentrations. The standard strain *Streptococcus pneumoniae* ATCC 45615 was the most effective with the extract's lowest MIC and MBC values. This study showed that nettle extract (*Urtica dioica*) would contribute to the research results in the scientific literature on its antimicrobial effect.

1. Introduction

Nettle, a member of the Urticaceae class, whose Latin name is *Urtica dioica*, has many important functions in traditional therapy due to its effects that support medical treatments. Various publications show that this plant is very effective in treating blood pressure, diabetes, and prostatic hyperplasia, rheumatoid arthritis, and allergic rhinitis [1]. Medicinal plants, also called herbs, are traditionally consumed as natural remedies to treat various diseases. The whole plant, parts of the plant, or extracts of these plants can be used. The nettle plant extract is used as a reducing agent for medicinal purposes in the synthesis stages of many nanoparticles [2-3]. Studies have also revealed that although chemical drugs are used successfully in treatments, they may show some side effects. Since natural products such as nettle do not accumulate in the body like other drugs, they have a biological balance and cause fewer side effects [4-5].

Urtica dioica is an endemic plant that grows mainly in tropical regions such as India and Malaysia, with 40 genera and about 500 species with medicinal value [3]. It also grows in Africa and Europe [3]. The leaves and roots of the nettle (*Urtica dioica* L.), also found in our country, are used for medicinal purposes. Among the *Urtica* species, *Urtica dioica* and *Urtica urens* are known in many parts of the world and have been consumed for ages as medicinal plant varieties [6]. Beschia et al. [7] reported that phenolic

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compounds contained in nettle plant extracts provide inhibitory activity against various types of microorganisms and can be effective in food preservation with these properties. When phenolic extract analyzes are examined, it is seen that they contain caffeic, ferulic, sinapic acid, and esculetin [8]. It also contains bioactive compounds such as flavonoids, polysaccharides, carotenoids, lignans, minerals (mostly iron), ascorbic acid, tannins, carvacrol, and thymol. These compounds are also used in the food packaging industry as they have antioxidant and antibacterial properties as an excellent bio preservative [9]. The compound that provides the essential antioxidant effect is quercetin, which accumulates in its leaves [10-11].

Plant extract from *Urtica dioica* L., methicillin-resistant *Staphylococcus*, vancomycin-resistant *Enterococcus*, *Escherichia coli*, *Klebsiella*, *Pseudomonas*, and imipenem-resistant *Acinetobacter* have bacteriostatic effects against some bacteria. They also show antifungal activity that suppresses the proliferation and development of *Aspergillus*, *Mucor* and *Candida albicans* [12].

While numerous studies highlight the beneficial effects of *Urtica dioica*, understanding the molecular mechanisms underlying these effects can uncover new horizons for new therapeutic strategies [13, 14]. Our study aims to evaluate the antibacterial activity of the extract obtained from the leaves of this valuable *U. dioica* against pathogenic microorganisms that mostly affect humans.

In this study, it was also planned to compare the antimicrobial activity of nettle extract obtained from the Erzincan region with the results of other studies.

2. Material and Method

2.1. Materials

Urtica diocia plants were collected from Büyük Çakırman (formerly Vank) village of Erzincan province in the Eastern Anatolia Region of Turkey in May 2021. Identification of the nettle was done by Assoc.Prof.Dr. Mustafa KORKMAZ, "Department of Biology, Faculty of Science and Arts, Erzincan Binali Yıldırım University, Erzincan, Turkey."

2.2. Preparation of Nettle Extract

After the nettle was collected, it was washed, dried in the shade, and turned into powder. The extract was prepared according to the method described by Holopainen et al. [15]. The dried leaves of the plant were extracted using 95% ethanol. The extract was kept at 4°C for two days and filtered through a 45-µm membrane filter. Finally, the extract was separated from the solvent using the evaporator. The extract was placed in dark glass bottles and stored at -20°C.

2.3. Preparation of Test Microorganisms and Detection of MIC and MBC by Broth Microdilution Test

For evaluation of antimicrobial activity, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 45615, *Klebsiella pneumoniae* ATCC 70063, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 standard strains were used. Clinical isolates of microorganisms were obtained from Erzincan Binali Yıldırım University, Faculty of Medicine Microbiology Laboratory. Standard strains were first transferred to a Brain Heart Infusion Broth (bioMerieux, France) medium, and afterward, they were incubated for one night. They were passaged into media with 5% sheep blood (bioMerieux, France) and then inoculated from fresh passages into Brain Heart Infusion Broth (bioMerieux, France) media. Standard strains inoculated into Brain Heart Infusion Broth (bioMerieux, France) media were prepared using a DensiCHEK™ Plus densitometer device (bioMerieux, France) at 0.5 McFarland turbidity standard ($1.5x10^8$ microorganisms in ml). Then, the number of microorganisms in the tubes was diluted to be $1.5x10^7$, $1.5x10^6$, $1.5x10^5$, $1.5x10^4$ cfu/ml with the dilution method. MIC and MBC values were investigated for four different bacterial concentrations. The broth microdilution method was used to investigate the antimicrobial effects of nettle extract. For antimicrobial tests, sterile microdilution plates with 96-well U-bottom wells were used. By making serial dilutions of the nettle extract at a concentration of 3mg/mL, whose antimicrobial activity was investigated, with the brain heart infusion broth (bioMerieux, France), concentrations of 3 mg/mL, 1.5 mg/mL, 0.75 mg/mL, 0.1875 mg/mL, 0.09375 mg/mL, 0.046875 mg/mL, 0.0234375 mg/mL were obtained. Standard strains diluted to 100 microliters in equal volume were added to the wells containing 100 microliters of the extract at different concentrations of $1.5x10^7$, $1.5x10^6$, $1.5x10^5$ and $1.5x10^4$cfu/m. In addition, the last three wells were designed as follows: only the extract was placed in the first well; the broth, which was the positive control with the pathogen added to the second well; and the brain heart infusion broth, which was used as the negative control, was placed in the last well. Then, the plates were incubated at 150 rpm at 35 degrees for 24 hours using a Heidolph brand shaking incubator device (Germany) (Figure 1). After the MIC values

were determined, 10 microliter passages were made on a 5% sheep blood agar medium and incubated for another 24 hours. MBC values were determined by detecting the last well without growth (Figure 2).

Figure 1. Incubation of nettle extracts in the shaking incubator

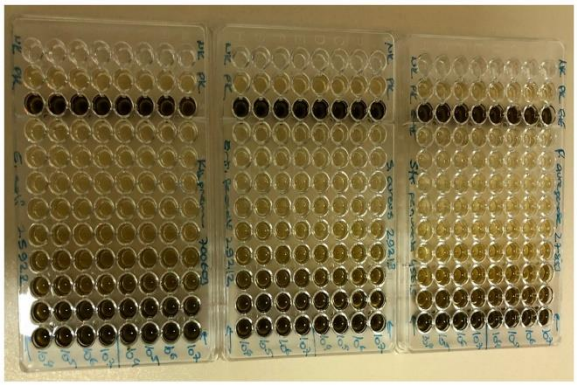

Figure 2. Plates used in MIC and MBC evaluation

3. Results and Discussion

3.1. MIC and MBC values of nettle extract

MIC and MBC breakpoint values obtained against six different standard strains are given in Table 1.

Table 1. MIC and MBC breakpoint values obtained against six different standard strains

Pathogen bacteria	Bacteria concentration (cfu/mL)	MIC value (mg/mL)	MBC value (mg/mL)
Staphylococcusaureus ATCC 29213	$1.5x10^7$	-	-
	$1.5x10^6$	0.375	1.5
	$1.5x10^5$	0.1875	0.75
	$1.5x10^4$	0.1875	0.75
Enterococcus faecalis ATCC 29212	$1.5x10^7$	1.5	3
	$1.5x10^6$	0.75	1.5
	$1.5x10^5$	0.75	1.5
	$1.5x10^4$	0.75	1.5
Streptococcus pneumoniae ATCC 45615	$1.5x10^7$	0.046875	0.375
	$1.5x10^6$	0.0234375	0.375
	$1.5x10^5$	0.0234375	0.1875
	$1.5x10^4$	0.0234375	0.09375
Klebsiella pneumoniae ATCC 70063	$1.5x10^7$	-	-
	$1.5x10^6$	-	-
	$1.5x10^5$	-	-
	$1.5x10^4$	-	-
Escherichia coli ATCC 25922	$1.5x10^7$	0.375	0.75
	$1.5x10^6$	0.375	0.75
	$1.5x10^5$	0.375	0.75
	$1.5x10^4$	0.1875	0.375
Pseudomonas aeruginosa ATCC 278536	$1.5x10^7$	-	-
	$1.5x10^6$	-	-
	$1.5x10^5$	1.5	-
	$1.5x10^4$	1.5	-

In the study, MIC and MBC values were investigated for four different bacterial concentrations. *Klebsiella pneumoniae* MIC and MBC values could not be found for ATCC 70063. No bacteriostatic or bactericidal effect had been observed for *Klebsiella pneumoniae* ATCC 70063 in the extract at the concentrations studied. MIC values were found for *Pseudomonas aeruginosa* ATCC 278536 only at $1.5x10^5$ and $1.5x10^4$ concentrations, and MBC values could not be determined; that is, the bacteriostatic effect could be detected only at $1.5x10^5$ and $1.5x10^4$ concentrations, while no bactericidal effect was observed. MIC and MBC values at various levels were found in the remaining standard strains; bacteriostatic and bactericidal effects were determined at multiple concentrations. The lowest MIC and MBC values at all concentrations were obtained for *Streptococcus pneumoniae* ATCC 45615. The effects of other standard strains, whose MIC and MBC values were determined, were also determined at various concentrations.

When the MIC values showing the bacteriostatic effect were examined, the lowest MIC obtained at the $1.5x10^7$, concentration was found, respectively, in *Streptococcus pneumoniae* ATCC 45615, *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212 standard strains. The lowest MIC values at the $1.5x10^6$ concentration was detected, respectively, in *Streptococcus pneumoniae* ATCC 45615, *Escherichia coli* ATCC 25922 = *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 standard strains. The lowest MIC values at the $1,5x10^5$ concentration were determined, respectively, *in Streptococcus pneumoniae* ATCC45615, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 278536 standard strains. The lowest MIC values at the $1,5x10^4$ concentration were detected in *Streptococcus pneumoniae* ATCC 45615, *Escherichia coli* ATCC 25922 = *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *and Pseudomonas aeruginosa* ATCC 278536 standard strains, respectively.

When the MIC values showing the bacteriostatic effect were examined, the lowest MIC obtained at the $1.5x10^7$, concentration was found, respectively, in *Streptococcus pneumoniae* ATCC 45615, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 standard strains. The lowest MIC values at the $1.5x10^6$ concentration was detected, respectively, in *Streptococcus pneumoniae* ATCC 45615, *Escherichia coli* ATCC 25922 = *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 standard strains. At $1.5x10^5$ the lowest MBC values were determined, respectively, in *Streptococcus pneumoniae* ATCC 45615 *Staphylococcus aureu*s ATCC 29213 = *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212 standard strains. At the $1.5x10^4$ concentration, the lowest MBC values were detected, respectively, in *Streptococcus pneumoniae* ATCC 45615, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 standard strains.

When the studies on the antibacterial effects of nettle extract were examined, it was seen that two methods were preferred as disk diffusion method and liquid microdilution method. In this study, the liquid microdilution method was used to obtain quantitative results to examine the nettle extract's antibacterial effect. Unlike other studies, MIC and MBC values were investigated for four different bacterial concentrations.

For comparison, when other studies conducted for the similar purposes as our study were examined in chronological order, Gülçin et al. [16], in their research with the disc diffusion method, have suggested that *Urtica dioica* aqueous extract is effective against *Escherichia coli* ATCC 9837, *Proteus mirabilis* (Clinical isolation), *Citrobacter koseri* (Clinical isolation), *Enterobacter aerogenes* (Clinical isolation), *Staphylococcus aureus* (ATCC 6538, *Streptococcus pneumoniae* (ATCC 49619) *Micrococcus luteus* (Clinical isolation), *Staphylococcus epidermidis* (clinical isolate) and *Candida albicans* (ATCC 10231) because they create a zone diameter. However, they reported

that no zone was formed against *Pseudomonas aeruginosa* (ATCC 9027), and therefore it was ineffective. Although the methods were different, when the results were evaluated, they were compatible with our study.

Dar et al. [17] examined the antibacterial activity of the extracts obtained by using hexane, chloroform, ethyl acetate, and methanol by the disk diffusion method. They found that no zone was formed with the extracts made using methanol and ethyl acetate, which were ineffective. Since they found the hexane extract to be effective, they investigated the MIC values with the tube dilution method and found 10^6 per milliliter concentrations of *E. faecalis, E. coli, K. pneumoniae, P. aeruginosa, S. aureus, S. flexneri,* and *S. typhi* that gave MIC values of 125, 15.62, 31.25, 250, 31.25, 125, and 7.81 μg/mL, respectively. Unlike our study, they found hexane extract to be effective against *K. pneumoniae* and *P. aeruginosa* strains. It is thought that this difference may be due to the different antimicrobial activity of the *Urtica dioica* plant grown in other regions.

In Kukric et al. [18], the antibacterial effects of *Urtica dioica* ethanolic extract were tested against the *Bacillus subtilis* strain isolated from food, *Lactobacillus plantarum*, *Pseudomonas aeruginosa* and *Escherichia coli*, and *Escherichia coli* isolated from urine. They used the macro dilution method against these bacteria in the study and reported that the ethanol extract of *Urtica dioica* leaf, diluted with methanol, showed a weak antibacterial effect on *B. subtilis* and *E. coli* isolated from food, yet no antibacterial activity was observed on *E. coli*, isolated from urine, or on *P. aeruginosa* and *L. plantarum* also isolated from food. Unlike the researchers' study, MIC values were obtained at some concentrations against *P. aeruginosa* in our research. However, it was thought that this might be due to different concentrations, different bacterial concentrations, and different antibacterial effects of extracts obtained from stinging nettles in other regions.

In Modarresi-Chahardehi et al. [19], they examined the antibacterial and antifungal effects of the extracts of *Urtica dioica* prepared with nine different solvents against 28 bacteria, three yeasts, and seven mold fungi; they reported that the extracts had antibacterial activities but did not show antifungal activity. When the bacterial dimension of the study was examined, it was observed that the results were generally similar to our study.

Ghaima et al. [20] investigated the antimicrobial activity of *Urtica dioica* leaf extract against five microorganisms: *Aeromonas hydrophila, Salmonella typhi, Staphylococcus aureus, Bacillus cereus,* and *Escherichia coli*, by the disc diffusion method. They reported that nettle extract was effective on these five bacteria.

Ramtin et al. [5] investigated the effect of alcoholic extract of nettle leaves on *B. cereus*, *S. aureus, K. pneumonia, P. aeruginosa, E. faecalis, E. coli* strains by the disc diffusion and liquid dilution methods. The extract is effective on all strains, and its results are consistent with our study.

Kulcu et al. [21] also investigated the MIC values of extracts made with different solvents of *Urtica dioica* against pathogens, and found the MIC value of the chloroform extract for *S. aureus* as 512 μg/mL, while the MIC value of the hexane extract for *E. faecalis* was >1024 μg/mL. Although the MIC values they obtained were different in our study, it was predicted that this might be due to different bacterial concentrations and the different antibacterial effects of the extracts obtained from nettles in different regions.

In the study conducted by Çolak et al. [22], it was determined that the MIC values for the ethanol extract determined for *Escherichia coli* (clinical isolate), *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* (clinical isolate), *Staphylococcus aureus* ATCC 29213, and MRSA (Methicillin-Resistant *Staphylococcus aureus*; clinical isolate) were found to be 2, 2, 2, 4, 4 mg/mL, respectively. When compared, the MIC values in our study were lower. It was thought that this result might be due to the different antimicrobial activity of the *Urtica dioica* plant grown in other regions and the difference in bacterial concentrations.

Kačániová et al. [11] determined in their study whether there is an antimicrobial effect in various plant extracts by the disc diffusion method. In addition, they investigated MIC50 and MIC90 values by the liquid microdilution

method for the strains that they detected effects by the disc diffusion method. They found MIC50 and MIC90 values of the *Urtica dioica* extract for *P. aeruginosa*, *E. faecalis*, *S. aureus* and *S. pneumoniae* to be 9.56 -10.24, 12.78-13.59, 7.42-8.82, and 8.53-9.54 µg/mL, respectively. In this study, *Urtica dioica* extract was found to be effective in terms of antimicrobials. The results were compatible with our study.

4. Conclusion and Suggestions

In our study, MIC and MBC values were determined for different concentrations of the extract obtained from the leaves of nettles collected from the Erzincan region, starting from 3 mg/mL, were prepared with serial dilutions $1.5x10.^{7}$, $1.5x10^{6}$, $1.5x10^{5}$ and $1.5x10^{4}$ cfu/ml at four different concentrations for pathogen standard bacterial strains. MIC and MBC values could not be determined for *K. pneumoniae*, while MIC values were determined only at two concentrations for *P. aeruginosa*. Since it was stated in the text above that some studies provided an effect against these strains, it is predicted that higher nettle extract concentrations and MIC and MBC values could be detected in our study. In this context, the determination of MIC and MBC values for different bacterial concentrations and nettle extracts at different concentrations will be useful to establish a common standard for comparative studies in which regional differences can be revealed. In addition, evaluating the bioactive compounds, they contain separately or together to understand the molecular mechanisms underlying the antimicrobial activity of *Urtica dioica* may open new horizons in terms of therapeutic strategies.

Contributions of the Authors

The contributions of each author to the article should be indicated.

Conflict of Interest Statement

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics.

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