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Assessment of the Antioxidant Effects of *Urtica dioica* Aqueous Extract on Free Radical Scavenging, Determination of Total Phenol and Flavonoid Content, and the Bioactive Compound Groups

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ABSTRACT

Medicinal plants have been used since ancient times as a source for treating diseases. Nettle (Urtica dioica) possesses anti-inflammatory, diuretic, antihistamine, antimicrobial, and immune-boosting properties.. The aim of this study was to investigate the antimicrobial effect of nettle leaf aqueous extract using agar disc diffusion, agar well diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) methods against a number of Gram-positive and Gram-negative bacteria. Results from agar disc diffusion and agar well diffusion tests showed that the most sensitive and resistant fungal strains to nettle leaf aqueous extract were *Staphylococcus aureus* and *Salmonella typhi*, respectively. As the concentration of nettle leaf extract increased, the zone of inhibition for all tested fungal strains significantly increased. The results indicated that the minimum inhibitory concentration for gram-negative bacteria such as *Escherichia coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa* was 128, 256, and 256 mg/ml, respectively, and for gram-positive bacteria including *Listeria innocua*, *Staphylococcus aureus*, and *Bacillus cereus* was 128, 64, and 128 mg/ml, respectively. The minimum bactericidal concentration for all tested strains was greater than 512 mg/ml. This study suggests that nettle leaf aqueous extract can be used as a natural antimicrobial compound in the food industry or in the treatment of certain infectious diseases.

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1- Introduction

The use of medicinal plants has a long history in the world, with 80% of developing countries still relying on traditional medicines derived from medicinal plants. The World Health Organization has also registered over 20,000 species of medicinal plants and described medicinal plants as a potential source of new drugs. There are over 1340 defined antimicrobial plants and over 30,000 antimicrobial compounds have been isolated from plants. In addition, it is estimated that 14-28% of higher plant species are medicinal and 74% of plant-based compounds with biological properties have been discovered based on medicinal uses [2-1].

Antibiotics are certainly one of the most vital therapeutic discoveries of the 20th century. On the other hand, only one-third of infectious diseases are treated with these synthetic drugs. Due to widespread misuse and overuse of antibiotics, antibiotic resistance has increased in recent years. Therefore, there is a growing demand for the development of new antimicrobial agents that can reduce antibiotic use and combat the development of resistance. This has led researchers to isolate and identify novel bioactive chemicals from plants to combat microbial resistance [3]. Also, considering that about 50% of current drugs and food-drugs are natural products and their derivatives [4], medicinal plants are one of the sources that have a wide range of biologically active compounds, but a large part of them have not yet been discovered. Currently, about one-third of commercial drugs contain at least one plant compound. Worldwide, medicinal plants and their compounds have been proposed as potential alternatives to antibiotics against infectious diseases [6-5].

In this context, plant extracts are sources of antimicrobial molecules. Due to their natural origin and generally good antimicrobial and biological properties, they enjoy high popularity among consumers. The increasing use of these extracts is due to several advantages, including reducing the incidence of diseases, environmental problems, and microbial resistance to chemical preservatives. Therefore, plant extracts can play an effective role in promoting human health and protecting the environment as a suitable alternative to antibiotics [7].

The Nettle plant, with the scientific name *Urtica dioica L.*, belongs to the Urticaceae family. It is an herbaceous perennial plant that can grow up to two meters tall and has prickly leaves. It grows in moist fields and meadows in shady areas. The Nettle plant is found in temperate regions of Europe, Asia, North Africa, and North America [9-8]. This plant has been used as a medicinal plant since ancient times and is still used in traditional medicine for a wide range of diseases. It also has anti-inflammatory, antimicrobial, and analgesic effects. In addition, it is proposed for the treatment of high blood pressure, rheumatism, arthritis, and heart disease. The Nettle plant contains minerals (Ca, Mg, Zn, Mn, Cu) and vitamins (provitamin A, carotenoids, B2, B5, B9, C, D, E, K) [11-10].

Moradi et al. (2017) demonstrated that the aqueous extract of the Nettle plant has both antibacterial and antifungal properties, suggesting that it can have an effect on a wide range of microorganisms [12]. In another study, the aqueous extract of Nettle was investigated and their results showed

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that this extract had antimicrobial activity against some Gram-positive and Gram-negative bacteria [13]. In addition, the antimicrobial activity of Nettle plant extract has been reported in various studies. In this present study, the potential antimicrobial effect of aqueous Nettle extract on a number of pathogenic microorganisms was investigated.

2- Materials and Methods

1-2 Chemicals and Microbial Strains

The materials used in this study were purchased from Merck, Germany. They included blank discs, triphenyltetrazolium chloride, Mueller-Hinton broth and agar culture media. The microbial strains used were *Escherichia coli* (ATCC 25922), *Listeria innocua* (ATCC 33090), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 14579), *Salmonella typhi* (ATCC 14028), and *Pseudomonas aeruginosa* (ATCC 27853). These strains were obtained from the microbial collection of the Department of Food Science and Engineering, Khuzestan University of Agriculture and Natural Resources.

2-2 Preparation of Aqueous Nettle Leaf Extract

To prepare aqueous Nettle leaf extract, the maceration method was used. For this purpose, the collected plant samples were dried in the shade and away from direct sunlight for 5 days after superficial washing, and were crushed with a mill before extraction. Then 50 g of leaf powder were added to 500 ml of distilled water and stirred for 24 h in a shaking incubator. The extracts were then passed through a filter and centrifuged at 5000 rpm for 10 min. The resulting precipitate was discarded and the supernatant phase containing the extract was collected. A vacuum rotary evaporator (model of the device) was used to remove the solvent, and finally the dried extracts were collected in dark glass containers and stored in the refrigerator until testing. It should be noted that the dried extract was sterilized using UV radiation [14].

2-3 Determination of Moisture Content and Water Activity

Before extracting Nettle leaves, the moisture content of the samples was determined. For this purpose, 1 gram of the sample was weighed and dried in an oven at 105 °C. The moisture content was calculated from the difference in mass before and after drying. The water activity (aw) of the dried Nettle leaf powder was determined using a water activity meter (model lab touch-aw, manufactured by novasina) [15].

2-4 Antimicrobial Activity Assessment of the Extract

2-4-1 Agar Disc Diffusion Method

In this method, different concentrations of the extract (64, 128, 256, and 512 mg/ml) were first prepared. Then, blank discs (6 mm diameter) containing 30 µL of each concentration of aqueous Nettle leaf extract were placed on the surface of Mueller-Hinton agar medium containing standard pathogenic strains (100 µL of microbial suspension) and stabilized and kept in the refrigerator for 15 minutes. Then they were incubated in an incubator at 37°C for 24 h. After the incubation period, the diameter of the zones of no growth around the discs was measured in millimeters and reported [16].

2-4-2 Agar Well Diffusion Method

To investigate the antimicrobial activity of aqueous Nettle leaf extract using the agar well diffusion method, the method of Alizada Behbahani et al. (2021) was used. In this method, 100 µL of microbial suspension was spread on Mueller-Hinton agar medium. Then, 6 mm diameter wells were created on the surface of the medium,

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60 µL of different concentrations of the extract were added to each well, and they were kept in the refrigerator for 15 min. Then they were incubated at 37 °C for 24 h in an incubator. After the incubation period, the diameter of the zones of no growth around the wells was measured in millimeters and reported [17].

2-4-3 Minimum Inhibitory Concentration (MIC)

To determine the minimum inhibitory concentration (MIC) of aqueous Nettle leaf extract, different dilutions of the extract (512, 256, 128, 64, 32, 16, 8, 4, 2, 1 mg/mL) were prepared using Mueller-Hinton broth. 100 µL of each dilution was added to wells of a 96-well microplate, and then 10 µL of microbial suspension was added. The microplate was incubated at 37°C for 24 h. After the incubation period, 10 µL of 5% triphenyltetrazolium chloride (TTC) solution was added to the wells and incubated for another 30 min. The first well in which no color change was observed was considered the MIC. It should be noted that Mueller-Hinton broth containing microbial suspension but no extract and Mueller-Hinton broth containing extract but no microbial suspension were used as positive and negative controls, respectively [18].

2-4-4 Minimum Bactericidal Concentration (MBC)

In this method, 100 µL of the wells of the 96-well microplate in which no color change was observed were inoculated onto Mueller-Hinton agar medium and incubated at 37°C for 24 h. The dilution in which no bacteria grew was considered the minimum bactericidal concentration (MBC) [19].

2-5- Statistical Analysis

The results of this study were analyzed using one-way ANOVA with SPSS version 18 software. The means of the data were compared using Duncan's test at a 95% confidence level ($p > 0.05$). Excel software was used to draw the graphs. All experiments were performed in triplicate.

3- Results and Discussion

3-1- Moisture Content and Water Activity

Nettle leaves were dried before being subjected to the extraction and evaluation of antioxidant and antimicrobial activity. The moisture content of the dried Nettle leaves was 76.8%, which indicated good quality and features for further analysis [20]. The water activity of dried Nettle leaves was 0.418, indicating a very low value that ensures the stability of their constituents. The drying process reduces the water activity of samples, inhibiting microbial growth and minimizing biochemical reactions that cause spoilage. Pathogenic bacteria cannot grow below a water activity of 0.85-0.86. Additionally, the drying process can change the microstructure of plant tissues and resulting to better extraction yields [21].

3-2- Antimicrobial Activity

The mean results of the inhibition zone diameters in the disc diffusion method are shown in Figure 1. The results showed that with increasing concentrations of Nettle leaf aqueous extract, the inhibition zone diameter for all studied pathogenic strains significantly increased. Discs containing the highest extract concentration (512 mg/ml) showed the largest inhibition zone diameter for *Staphylococcus aureus* and the smallest inhibition zone diameter for *Salmonella typhi*. The inhibition zone diameters for them were 21.37 mm and 16.31 mm, respectively, indicating the high sensitivity and resistance of these bacteria to the extract. In general, Gram-positive

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bacteria *Bacillus cereus*, *Listeria innocua*, and *Staphylococcus aureus* (Gram-positive bacteria) had larger inhibition zone diameters against Nettle aqueous extract compared to Gram-negative bacteria *Salmonella typhi* and *Pseudomonas aeruginosa* (Gram-negative bacteria). The high antimicrobial activity of the extract against Gram-positive bacteria compared to Gram-negative bacteria suggests that Gram-negative bacteria have a strong and complex outer cell membrane (lipopolysaccharide), while Gram-positive bacteria lack a complex membrane. It also appears that the speed and solubility of the antimicrobial agent in the lipid part of the cell membrane contribute to the resistance of microbial cells [22-23]. Noshad et al. (2021) investigated the antimicrobial activity of Nettle leaf ethanol extract against a number of Gram-positive and Gram-negative bacteria. The results of their study showed that Gram-positive bacteria were more sensitive to Nettle ethanol extract compared to Gram-negative bacteria [20]. Hamzezade Nakhjavani and Emam-Djomeh (2023) showed that the antimicrobial effect of Nettle alcoholic extract in edible film was significantly more effective ($p < 0.05$) against *Staphylococcus aureus* than *Escherichia coli* [24]. Another study reported that Nettle extract has antimicrobial properties against *Escherichia coli* and *Staphylococcus epidermidis* [25]. Zohra et al. (2021) investigated the antimicrobial properties of aqueous, ethanol, and acetone extracts of Nettle plant on four bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus*, and *Enterococcus faecalis*) using the disc diffusion method. The results of their study showed that ethanol and acetone extracts had inhibitory effects on bacterial strains with different inhibition zone diameters ranging from 10 to 16 mm. Among the strains, *Micrococcus luteus* was more sensitive to acetone extract, while aqueous extract showed no inhibitory effect on the bacterial strains [26]. In another study, the antimicrobial effect of methanol and ethanol extracts of Nettle plant was investigated on a number of pathogenic strains. The results showed that the largest inhibition zone diameter for both extracts was for Gram-positive bacteria and the smallest inhibition zone diameter was for Gram-negative bacteria [27].

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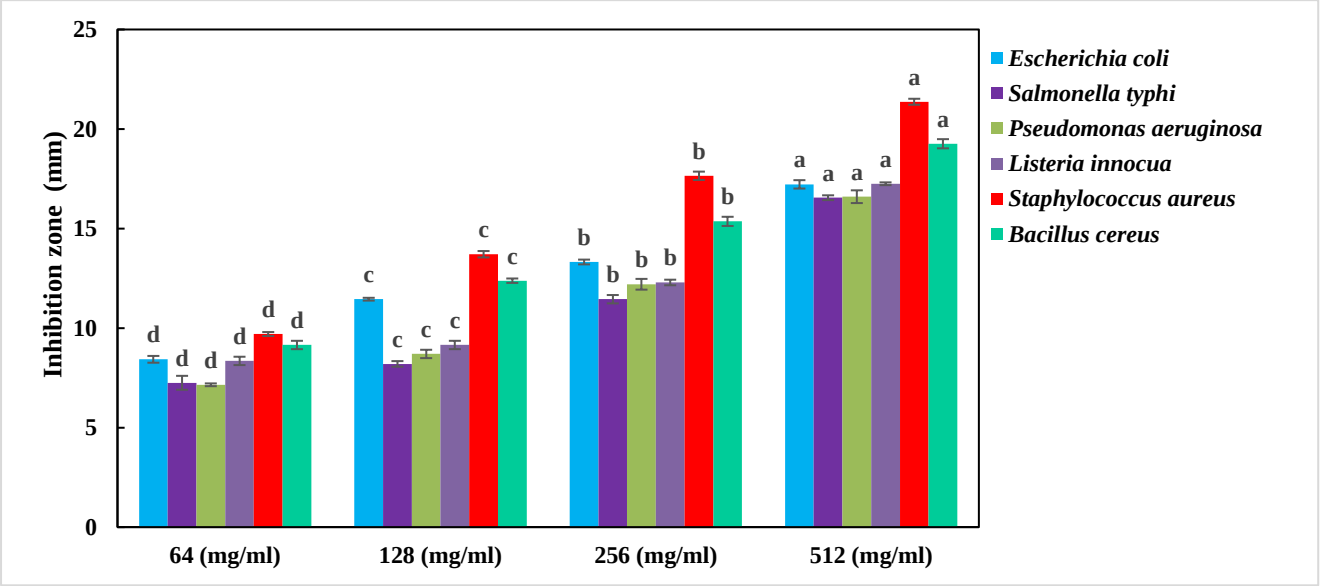

Fig.1. Antibacterial effect of Nettle (*Urtica dioica* L.) leaf aqueous extract by disk diffusion method.

Figure 2 presents the results of the mean inhibition zone diameters in the agar well method. The results showed that in the agar well method, with increasing concentrations of aqueous extract (64-512 mg/ml), the inhibition zone diameter significantly (at the 5% level) increased. The smallest inhibition zone diameter was observed for *Salmonella typhi* at a concentration of 64 mg/ml with an inhibition zone diameter of 7.38 mm, and the largest inhibition zone diameter was observed for *Staphylococcus aureus* at a concentration of 512 mg/ml with an inhibition zone diameter of 21.6 mm. In addition, the results of the antimicrobial activity of Nettle aqueous extract by the agar well method were slightly higher compared to the disc diffusion method. This is because in the agar well method, the antimicrobial agent (essential oil or extract) is in direct contact with the pathogenic strain, while in the disc diffusion method, the antimicrobial agent exerts its effect on the pathogenic strain after passing through the disc plates [18]. In a study by Roshani et al. (2016), the antimicrobial activity of ethanol and acetone extracts of Nettle leaves was investigated. They stated that both extracts had high inhibitory power against pathogenic strains [28]. Another study showed that alcoholic extract has antimicrobial activity against *Bacillus cereus* and *Vibrio parahaemolyticus* bacteria [29]. Saleh et al. (2014) investigated the antibacterial activity of aqueous and 95% ethanol extracts of Nettle leaves on some Gram-negative and Gram-positive bacteria using the agar well diffusion method. The bacteria used in this experiment were: *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella spp*., *Bacillus subtilis*, *Proteus spp*., *Salmonella spp*., They reported that both extracts had different antibacterial activities, and due to the higher solubility of active ingredients in ethanol extract compared to aqueous extract, ethanol extract was superior. *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella spp*. showed the highest sensitivity to the antibacterial effect of Nettle extract, while *Escherichia coli*, *Pseudomonas*, and *Proteus* were less

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sensitive, and only *Klebsiella spp.* was identified as resistant [30]. In addition, Kiaei et al. (2010) reported the antimicrobial effect of aqueous and methanolic extracts of Nettle root and leaf on *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [31]. Comparison of the results of this study with the findings of the present study showed that *Staphylococcus aureus* was also the most sensitive microbial strain. Therefore, it can be stated that the results of these researchers are in accordance with the findings of the present study.

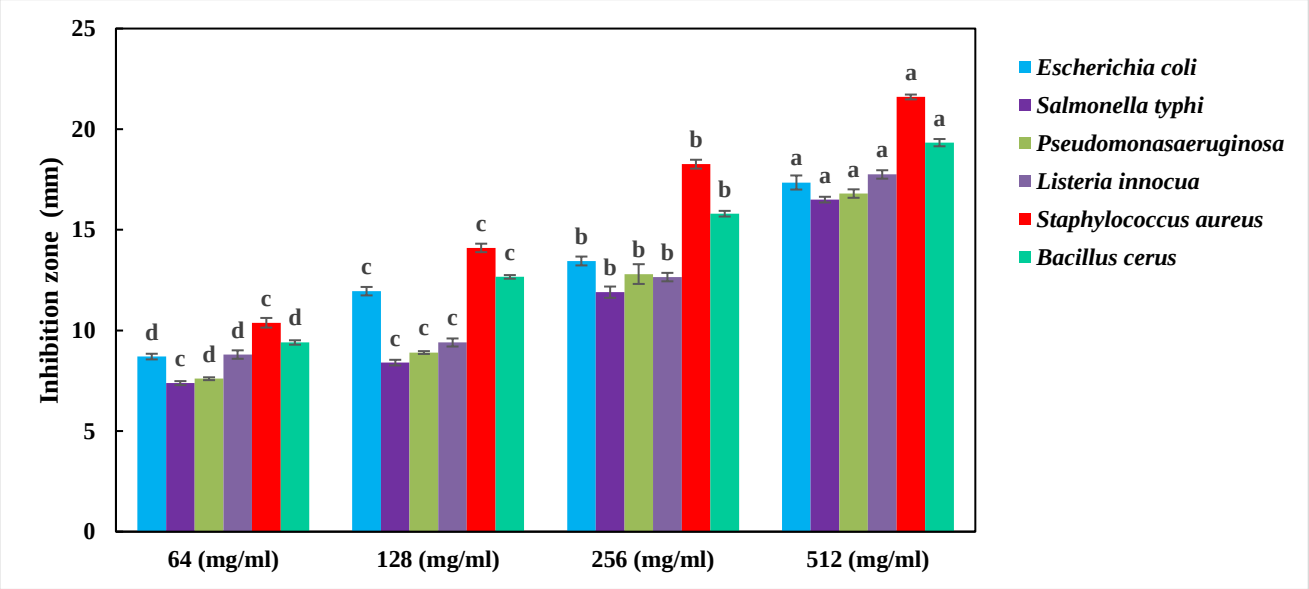

Fig. 2. Antibacterial effect of Nettle (*Urtica dioica* L.) leaf aqueous extract by well diffusion agar method

The antimicrobial results of Nettle aqueous extract based on minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against pathogenic strains are presented in Table 1. According to the results, the MIC for Gram-negative bacteria, including *Escherichia coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa*, was 128, 256, and 256 mg/ml, and 256 mg/ml, respectively, and for Gram-positive bacteria, including *Listeria innocua*, *Staphylococcus aureus*, and *Bacillus cereus*, it was 128, 64, and 128 mg/ml, respectively. Therefore, the highest level of inhibition was for the Gram-positive species *Staphylococcus aureus* and the lowest level was for the Gram-negative species *Salmonella typhi* and *Pseudomonas aeruginosa*. A study investigated the antimicrobial effect of aqueous extracts of several plants, including Nettle, on *Staphylococcus aureus* bacteria. Their results showed that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for this species were 678.5 and 1375, respectively [32]. Moradi et al. (2017) investigated the antibacterial and antifungal activity of aqueous and alcoholic extracts of Nettle plant on the bacteria *Staphylococcus aureus*, *Proteus vulgaris*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, and the yeast *Candida albicans*. The results of this study showed that aqueous extracts had the greatest antibacterial effect compared to alcoholic extracts. Also, the Gram-positive bacteria *S. aureus* and *L. monocytogenes* were the most sensitive bacteria in the aqueous extract, and the

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Gram-negative bacteria *K. pneumoniae* and *P. vulgaris* showed the best antimicrobial effect. In addition, the aqueous extract of Nettle plant showed antifungal activity against the fungus C. albicans [12]. In a study conducted by Jafari et al. (2012), the antibacterial properties of different extracts of Nettle plant were investigated. The results showed that ethanol extracts from different parts of this plant have antimicrobial activity against some bacteria, including *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*. Among these extracts, Nettle seed extract had the greatest inhibitory effect on Gram-positive bacteria, and Nettle leaf extract had the greatest effect on Gram-negative bacteria. In addition, aqueous extracts of this plant had a positive effect on the growth of all microorganisms except *Pseudomonas aeruginosa* [33]. Motamedi et al. (2014) showed that the minimum inhibitory concentration (MIC) of Nettle ethanol extract against *Staphylococcus epidermidis* and *Escherichia coli* was 10 and 40 mg/ml, respectively. The MIC of methanol extract against *Staphylococcus* aureus and *Staphylococcus epidermidis* was 40 and 10 mg/ml, respectively. The minimum bactericidal concentration (MBC) was only obtained for *Staphylococcus epidermidis*, which was 20 mg/ml [27]. The results of the present study were consistent with other studies conducted on the antimicrobial effect of essential oils and plant extracts [34-46].

Table 1. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Nettle (*Urtica dioica* L.) leaf aqueous extract on pathogenic bacteria

Microorganism	MIC (mg/mL)	MBC (mg/mL)
Escherichia coli	128	>512
Salmonella typhi	256	>512
Pseudomonas aeruginosa	256	>512
Listeria innocua	128	>512
Staphylococcus aureus	64	>512
Bacillus cereus	128	>512

4- Conclusion

The results of this study showed that with increasing concentration of Nettle leaf aqueous extract, the diameter of growth inhibition zones increased for Gram-positive bacteria compared to Gram-negative bacteria studied. Therefore, it can be stated that Nettle plant aqueous extract has significant antimicrobial potential that can be used in the development of herbal medicines and natural compounds to combat various infections. These properties may be effective against the bacteria *Escherichia coli*, *Listeria innocua*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi*, and *Pseudomonas aeruginosa*. However, further research is needed on other pathogenic strains under in vitro conditions.

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مقاله علمی–پژوهشی

ارزیابی فعالیت ضدمیکروبی عصاره آبی گیاه گزنه (*Urtica dioica*) بر تعدادی از باکتری‌های گرم مثبت و گرم منفی در شرایط آزمایشگاهی

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چکیده

گیاهان دارویی از دیرباز به عنوان منبعی برای درمان بیماری‌ها مورد استفاده قرار گرفته‌اند. گیاه گزنه (*Urtica dioica*) دارای خواص ضدالتهابی، دیورتیک، آنتی‌هیستامین، ضد میکروبی و تقویت کننده سیستم ایمنی است. هدف از این مطالعه، بررسی اثر ضدمیکروبی عصاره آبی برگ گزنه با روش‌های دیسک دیفیوژن آگار، چاهک آگار، حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی بر تعدادی از باکتری‌های گرم مثبت و گرم منفی در شرایط آزمایشگاهی بود. نتایج آزمون‌های دیسک دیفیوژن آگار و چاهک آگار نشان داد که حساس‌ترین و مقاوم‌ترین سویه بیماری‌زا نسبت به عصاره آبی برگ گزنه *استافیلوکوکوس اورئوس* و *سالمونلا تیفی* می‌باشند. با افزایش غلظت عصاره برگ گزنه قطر هاله عدم رشد برای تمامی سویه‌های بیماری‌زا مورد بررسی به‌طور معنی‌داری افزایش یافت. نتایج نشان داد که حداقل غلظت مهارکنندگی برای باکتری‌های گرم منفی از جمله *اشرشیا کلی*، *سالمونلاتیفی* و *سودوموناس ائروژینوزا* به ترتیب ۱۲۸، ۲۵٦ و ۲۵٦ میلی‌گرم بر میلی‌لیتر و برای باکتری‌های گرم مثبت از جمله *لیستریا اینوکوا*، *استافیلوکوکوس اورئوس* و *باسیلوس سرئوس* به ترتیب ۱۲۸، ٦٤ و ۱۲۸ میلی‌گرم بر میلی‌لیتر بود. نتایج حداقل غلظت کشندگی برای همه سویه‌های مورد بررسی بزرگتر از ۵۱۲ میلی‌گرم بر میلی‌لیتر بود. مطالعه حاضر نشان داد که می‌توان از عصاره آبی برگ گزنه به عنوان یک ترکیب ضدمیکروبی طبیعی در صنعت غذا بهره برد.

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