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RESEARCH ARTICLE

In Vitro Antibacterial Effect of Hesperidin Microemulsion to *Staphylococcus aureus* and *Listeria monocytogenes*

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ABSTRACT

Foodborne bacterial infections and intoxications constitute a large proportion of bacterial diseases worldwide. In recent years, there has been an increasing resistance to chemotherapeutics used in the treatment of these diseases. Studies have shown that some compounds extracted from natural products and plants have alternative potential to antibiotics. In this study, it was aimed to reveal the antimicrobial potential of hesperidin microemulsion on *Listeria monocytogenes* and *Staphylococcus aureus*. In the study, the broth microdilution method was used to demonstrate *in vitro* antimicrobial activity. In the microdilution test, the MIC values of hesperidin for *S. aureus* and *L. monocytogenes* were as 128 µg/mL. In comparison, the MIC values of ampicillin for *S. aureus* and *L. monocytogenes* were as 0.5 µg/mL. Hesperidin is a compound with biologically valuable properties. Its antimicrobial effect may open a promising field as an alternative to antibiotics. However, detailed studies on hesperidin and similar potential active substances are needed to fully understand its therapeutic potential and to define its mechanisms of action.

Keywords: Antibacterial, Hesperidin, *Listeria monocytogenes,* MIC, *Staphylococcus aureus*

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Hesperidin Mikroemülsiyonunun *Staphylococcus aureus* ve *Listeria monocytogenes*'e Karşı *In Vitro* Antibakteriyel Etkisi

Gıda kaynaklı bakteriyel enfeksiyonlar ve zehirlenmeler dünya genelinde bakteriyel hastalıkların büyük bir bölümünü oluşturmaktadır. Son yıllarda bu hastalıkların tedavisinde kullanılan kemoterapötiklere karşı artan bir direnç söz konusudur. Yapılan çalışmalar, doğal ürünlerden ve bitkilerden elde edilen bazı bileşiklerin antibiyotiklere alternatif potansiyele sahip olduğunu göstermiştir. Bu çalışmada hesperidin mikroemülsiyonunun *Listeria monocytogenes* ve *Staphylococcus aureus* üzerindeki antimikrobiyal potansiyelinin ortaya konulması amaçlanmıştır. Çalışmada, *in vitro* antimikrobiyal aktiviteyi göstermek için broth mikrodilüsyon yöntemi kullanılmıştır. Mikrodilüsyon testinde hesperidinin *S. aureus* ve *L. monocytogenes* için MİK değerleri 128 µg/mL olarak bulunmuştur. Buna karşılık, ampisilinin *S. aureus* ve *L. monocytogenes* için MİK değerleri 0.5 µg/mL olarak bulunmuştur. Hesperidin biyolojik olarak değerli özelliklere sahip bir bileşiktir. Antimikrobiyal etkisi antibiyotiklere alternatif olarak umut verici bir alan açabilir. Bununla birlikte, terapötik potansiyelini tam olarak anlamak ve etki mekanizmalarını tanımlamak için hesperidin ve benzeri potansiyel aktif maddeler üzerinde ayrıntılı çalışmalara ihtiyaç vardır.

Anahtar Sözcükler: Antibakteriyel, Hesperidin, *Listeria monocytogenes*, MİK, *Staphylococcus aureus*

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INTRODUCTION

Infectious diseases are generally caused by waterborne and foodborne microbial agents. Among these, pathogenic bacteria such as *Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes, Campylobacter* spp. and *Escherichia coli* O157:H7 are the leading causes of foodborne gastroenteritis (Bailey et al. 2003; Hizlisoy et al. 2020). *S. aureus* is a pathogen that is often blamed for a variety of serious diseases such as cellulitis, endocarditis, and bacteremia from skin infections (Pal et al. 2023). *S. aureus* is commonly found on the skin and mucous membranes of farm animals. The causative bacterial agent causes poisoning from food prepared from the products of animals produced as food worldwide (Narayan et al. 2023). *L. monocytogenes* is also considered one of the most important zoonotic agents as it causes disease in both animals and humans. *L. monocytogenes* is found in nature, food, humans, animals, and plants (Lourenco et al. 2022). *L. monocytogenes* infections in humans are primarily caused by the consumption of contaminated food, leading to listeriosis, which is characterized by life-threatening meningitis, septicemia, and premature stillbirths (Schaefer et al. 2022). Cross-contamination has been recognized as the primary cause of contact of *L. monocytogenes* with food (Dos Santos et al. 2021). Macrolide, quinolone, tetracycline, gentamicin and beta-lactam group antibiotics are generally used in the treatment of foodborne bacterial infections (Ge et al. 2022). Resistance to these antibiotics is a serious global public health problem. In recent years, there has been an increasing development of resistance to many of the currently used antibiotics (Majumder et al. 2020). Alternatively, it is important to use compounds found in nature or obtained by various methods such as extraction as effective antibacterial agents (Guglielmi et al. 2020). Here, not only antimicrobial resistance, but also the easy availability of natural substances, their cheapness, the lack of technological infrastructure in undeveloped and developing countries, and negative thoughts about the side effects of synthetic substances are effective (Baytop 1999). In terms of natural antimicrobial compounds, plants occupy an important place (Ju et al. 2022). Alkaloids, terpenes and phenolic compounds, also called secondary metabolites, are substances found in plants that are known to have antimicrobial properties (Duraipandiyan et al. 2006). Among the phenolic compounds, flavonoids are widely found in plants. Flavonoids can also be found in various parts of plants, especially in fruit peels, leaves, flowers and seeds. In addition, there are subclasses such as flavonols, flavones, isoflavones, anthocyanins and catechins. Each subclass shows structural differences and may be specific to certain plant groups (Duraipandiyan et al. 2006).

Active metabolites in plants are obtained from plants by extraction methods. Extraction is the method used to separate a targeted substance from a mixture, concentrate an ingredient, or remove the desired substance from a material by physical and chemical methods (Jha and Sit 2022). Some common plant extraction methods include cold extraction, hot extraction, maceration, supercritical carbon dioxide extraction, ultrasonic extraction, and sublimation. Which extraction method to use may vary depending on the targeted active ingredient, plant material, and other factors. Furthermore, the solvent used and extraction times are also important factors influencing the results (Mathews et al. 2024).

Hesperidin (C16H14O6 3',5,7-trihydroxy-4'-methoxy flavanone), also a herbal extract, is a type of flavonoid and especially found in citrus peels and fruit juice. It is usually found in high amounts in citrus fruits such as lemons, oranges, tangerines, and grapefruits (Garg et al. 2001). Hesperidin has antioxidant properties with a high amount of flavonoids, and thanks to these properties, it can protect cells against the harmful effects of free radicals (de Souza et al. 2022). Besides, some studies also report that hesperidin may support cardiovascular health. In particular, hesperidin may increase blood vasodilation, which in turn may regulate blood pressure (Valls et al. 2021). Hesperidin has also the potential to regulate cholesterol levels (Altunayar-Unsalan et al 2022). In some studies, it has been shown that hesperidin can inhibit the development of cancer cells (Tan et al. 2020; Hermawan et al. 2021). However, research in this area is incomplete, and more studies are needed to determine whether they have similar effects in humans (Önder et al. 2023). Moreover, some studies have suggested that hesperidin has the potential to regulate blood sugar levels. (Shams-Rad et al. 2020).

In our study, it was aimed to investigate the *in vitro* antibacterial activities of hesperidin active ingredient on *S. aureus* and *L. monocytogenes*, which are important food pathogens.

MATERIALS and METHODS

Materials

The active ingredient hesperidin (Sigma-Aldrich Catalogue no.520-26-3), which was studied for the demonstration of antibacterial activity *in vitro*, was commercially available. Stock solutions of hesperidin were prepared in 100 mg/mL with DMSO (Dimethyl Sulfate Oxide) dilutions. The antibacterial properties of the active ingredient were compared with ampicillin (A9518, Merck, Germany) (Karayıldırım 2017). Again, *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 standard strains were used to determine antibacterial activity.

Bacterial Inoculum

To determine the antibacterial activity of hesperidin, *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 were used. The isolates were inoculated into blood agar with 7% sheep blood added (Merck, Germany Catalog number: 103879) and left to incubate at 37°C for 18-24 hours. The reference strains were obtained from the culture collection of Erciyes University Faculty of Veterinary Medicine, Food Hygiene and Technology Laboratories.

Minimum Inhibitory Concentration (MIC) Test

A broth microdilution test was performed to demonstrate the antimicrobial efficacy of hesperidin against test bacteria (NCCLS 2007). In this method, briefly; at the end of 24 hours of incubation in an aerobic environment at 37°C, the fresh culture was inoculated into Mueller-Hinton broth (Merck, Germany) and the bacterial suspension was adjusted according to the 0.5 McFarland (~1.5x10^8 cfu/mL) standard was added to 100 µL equal volumes of 96-well microtiter plates. Prepared hesperidin stock solutions were added to 96-well microtiter plates and 1/2 serial dilutions were made. The dose was adjusted so that the hesperidin concentration in the wells ranged from 256 to 0.5 µg/mL. In the last well, 100 µL of liquid medium and 100 µL of bacterial suspension were used as negative control. All plates were covered with a sterile plate lid and incubated at 37 °C for 24 hours. The existing suspensions in the wells were planted in Mueller Hinton agar and left for incubation.

Wells containing dilutions of the ampicillin (A9518, Merck, Germany) as a positive control were adjusted to range from 4 to 0.36 µg/mL and were incubated in the same way. Additionally, controls were established for sterility, microorganism viability, and inhibitory activity of DMSO.

Statistical Analysis

The results of MIC values of Hesperidin against to *S. aureus* and *L. mononocytogenes* were expressed in mean+standard deviation using Microsoft Office Excel 2019 (Microsoft Corporation, Redmond, WA, USA). Significant differences between the averages were determined by the T-test (SPSS for Windows 11)

RESULTS

According to the results of the microdilution test, the MIC values of *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 bacteria were determined as 128 µg/mL and 128 µg/mL for hesperidin. In addition to these results, the MIC values of *S. aureus* and *L. monocytogenes* bacteria were determined as 0.5 µg/mL for ampicillin, respectively (Table 1). As a result of the T test, it was seen that there was no significant difference in terms of the MIC values of the bacteria.

Table 1. MIC values of hesperidin and ampicillin against *S. aureus* and *L. monocytogenes*

ATCC	Hesperidin	Ampicillin
S. aureus	128 µg/mL	0.5 µg/mL
L. monocytogenes	128 µg/mL	0.5 µg/mL

DISCUSSION

In recent years, the use of natural products in the treatment of infectious diseases has been increasing (Newman and Cragg, 2020). Among the main reasons for this increase are the side effects of synthetic drugs and the antimicrobial resistance of microorganisms against these drugs (Malekzadeh et al. 2001, Nostro et al. 2005). Around the world, interest in research examining the antimicrobial properties of extracts from plants has increased in parallel (Chassagne et al. 2021). Many plants such as *Alpinia galanga, Anethum graveolens, Asperugo procumbens, Bixa orellana, Feoniculum vulgare, Phyllanthus emblica* and *Vitis vinifera* have been examined for their antimicrobial properties around the world and in our country (Ozen et al. 2020). Alkaloids, terpenes, and phenolic compounds in medicinal plants are compounds that show antimicrobial activity in plants (Duraipandiyan et al. 2006). Hesperidin, a flavanone compound, is of medical importance due to its antioxidant, anti-inflammatory and antibacterial properties. Pharmacological studies have shown that hesperidin is a potent antimicrobial, analgesic, and immunomodulatory agent (Tejada et al. 2018).

In the study conducted by Abuelsaad et al. (2013), the antimicrobial activity of hesperidin against *Aeromonos hydrophila* was tested by broth microdilution method and as a result of the examination, it was revealed that hesperidin was active against *A. hydrophila* at doses of 100, 50, 25 and 12.5 mg/mL. In the studies of Ghorab and Ibraheim (2018), the antibacterial activity of hesperidin on *Streptococcus mutans* was investigated by agar diffusion method and the presence of an inhibition zone against bacteria was revealed at the end of the test.

In the study conducted by Karayıldırım (2017) in Turkiye, hesperidin microemulsion was tested in terms of antibacterial activity against various Grampositive and negative bacteria. At the end of the study, it was stated that hesperidin showed strong activity on *E. coli* (8 µg/mL) and *E. faecalis* (16 µg/mL) at a concentration equal to standard antibiotic gentamicin. Again, in the study conducted by Çetinkaya et al. (2019), it was reported that the application of hesperidin to patients with acute otitis media for 14 days reduced the symptoms of the disease with an effect similar to antibiotics.

In our study, according to the results of the MIC test performed to reveal the antimicrobial activity of hesperidin, the MIC values of *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 bacteria were found to be 128 µg/mL for hesperidin. In addition, MIC values of *S. aureus* and *L. monocytogenes* bacteria were determined as 0.5 µg/mL for ampicillin. Accordingly, although it is understood that the antibiotic ampicillin is effective at a lower titer, it is important that hesperidin, which is a natural product, also has antimicrobial activity.

The efficacy of its active compounds in plants varies depending on the collection, drying, and storage processes (Baytop 1999, Zaidan et al. 2005). There is a period when the pharmaceutical active compound of each plant is at the highest level (Baytop 1999). Therefore, plants should be harvested during periods when the active ingredient they contain is highest (Atata et al. 2003). The herbal active ingredient used in the study is a commercial microemulsion and is not affected by external factors to which other herbal metabolites are exposed if stored under appropriate conditions.

CONCLUSION

In conclusion, hesperidin is a biologically valuable compound. Its antimicrobial activity is a promising situation for alternative drug research to antibiotics. However, in order to discover its full therapeutic potential and to define its mechanisms of action in the later stages, detailed studies are needed on active ingredients with high potential as an alternative to hesperidine and similar antibiotics.

Conflict of interest: The author has no conflicts of interest to report.

Authors' Contributions: HH contributed to the project idea, design, execution, data acquisition and analysis of the study.

Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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