

Isorhamnetin as a promising natural bioactive flavonoid: *in vitro* assessment of its antifungal property

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Citation: Unver, T. (2024). Isorhamnetin as a promising natural bioactive flavonoid: *in vitro* assessment of its antifungal property. International Journal of Agriculture, Environment and Food Sciences, 8(1), 54-61

Received: November 17, 2023

Accepted: January 13, 2024

Published Online: March 25, 2024

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Available online at
<https://dergipark.org.tr/jaefs>
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Abstract

Isorhamnetin (3'-methylquercetin) is an important flavonoid produced as a secondary metabolite from medicinal and aromatic plants due to its pharmacological and therapeutic properties. Thus far, the anticancer, antiallergic, antiinflammatory, antioxidant, antiviral, and antimicrobial properties of isorhamnetin have been evaluated in indirect studies conducted with isorhamnetin found in plant extracts or essential oils or direct studies performed with pure isorhamnetin. However, this is the first study in the literature on the antifungal activity of 97% pure isorhamnetin against *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* using two assays including agar dilution and broth microdilution methods. This study showed that isorhamnetin has a significant inhibitory effect against all *Candida* species used. The minimum inhibitory concentration (MIC) value of isorhamnetin against *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* was 1.875 mg/mL, the same for all yeast strains. These results have opened a new horizon regarding the usability of isorhamnetin as a pharmacological therapeutic antifungal agent.

Keywords: Isorhamnetin, Flavonoids, Antifungal Activity, Pharmaceutical compound, Therapy

Introduction

The severity of fungal diseases varies depending on the type of yeast and the area of the body infected. *Candida* species affects the cutaneous layer and mucous membrane and causes infections in the mouth, skin, vagina, and intestines (Talapakko et al., 2021; Odds, 1994). If the host immune system is not functioning properly, *Candida* infection can spread to other surrounding areas of the heart or brain, causing severe symptoms and invasive candidiasis (Kuran, 2021; Pappas et al., 2018). If it is not controlled, *Candida* penetrates the bloodstream by breaking down the intestinal walls in the digestive system. It can cause leaky gut syndrome by releasing the toxins it synthesizes (Kuran, 2021). The proportion of non-*albicans* *Candida* species among *Candida* species that cause fungal diseases is increasing yearly and constitutes 35-65% of all fungal diseases. (Krcmery and Barnes, 2002). The most common non-*albicans* *Candida* species are *C. parapsilosis*, with a rate of 20-40%; *C. tropicalis*, with a rate of 10-30%; and *C. krusei*, with a rate of 10-35%. While the mortality rate due to *C. albicans* is around 20-40%, the mortality rate due to non-*albicans* *Candida* species is between 15% and 35% (Krcmery & Barnes, 2002).

A study reported that more than \$7.2 billion was spent on treating fungal diseases in the United States between 2005 and 2014, and *Candida* infections constitute the most crucial part of fungal diseases (Benedict et al., 2019). In Türkiye, the frequency of fungal infections in hospitalized patients has increased

significantly in the last 20 years (Karakoç, 2019). Fungal infection was detected in 12.5% of hospitalized patients, including intensive care units. 1/3 of fungal infections were isolated from the intensive care unit (Özçetin et al., 2009). The treatment process of fungal disease is carried out with intensive antibiotic and antifungal treatment. It is known that long-term antibiotic and antifungal use causes harm to the body (Benitez & Carver, 2019). The antibiotics used may cause side effects such as allergic symptoms such as itching and rashes on the skin, difficulty breathing, sudden low blood pressure, rapid heartbeat, and loss of consciousness. Digestive system disorders such as diarrhea, constipation, nausea, vomiting, bloating, indigestion, loss of appetite, and abdominal pain are among the harmful effects of antibiotics (Ekici, 2023). These are chemical treatment agents that have therapeutic properties but are not natural. Antifungal drug resistance is a significant problem in many fungal diseases, and its incidence in medical centers has increased in recent years (Fong, 1996; Cowen et al., 2014). For all these reasons, researchers have focused on natural antifungal substances. Plant-derived natural substances used in traditional medicine have been preferred due to their easy accessibility, cheapness, and widespread availability.

In traditional medicine, plant extracts from medicinal and aromatic plants or phenolic substances from these plants have been used for centuries. Medicinal plants are used in primary health care for their antimicrobial, anticancer, antithrombotic, antidiabetic, and many disease-therapeutic properties (Gong et al., 2020; Senizza et al., 2020; Man et al., 2019; Knezevic et al., 2016). Isorhamnetin is an important compound obtained from some of these medicinal plants (Ren et al., 2019; Pengfei et al., 2009; Teng et al., 2006). Isorhamnetin (molecular formula: $C_{16}H_{12}O_7$), called 3'-methylquercetin, is a natural compound and flavonoid with high pharmaceutical value. Isorhamnetin is a monomethoxyflavonol produced as a secondary metabolite, especially from medicinal and aromatic plants (Zou et al., 2023; Gong et al., 2020). Studies have reported that Isorhamnetin has antioxidant activity in the vascular smooth muscle and atrium of experimental animals, as well as cytoprotective and cardiovascular activity (Li et al., 2022; Xu et al., 2020; Kuti, 2004; Ibarra et al., 2002). It has been proven that isorhamnetin has pharmacological effects on various cancer cells (Hu et al., 2015; Li et al., 2014; Kim et al., 2011; Jaramillo et al., 2010). Isorhamnetin obtained from the plant source has proven anticancer activity against hepatocellular carcinoma cells and has been shown to induce cell cycle arrest in the G1 phase (Teng et al., 2006). Furthermore, isorhamnetin can prevent Alzheimer's disease and has effective pharmacodynamics against hyperuricemia and pulmonary fibrosis (Ishola et al., 2019; Adachi et al., 2019; Zheng et al., 2019). There are many pharmacological studies about isorhamnetin in the literature, but this is the first study of the antifungal activity of pure isorhamnetin against *Candida* species. This study investigated the antifungal activity of 97% pure isorhamnetin against *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* using agar dilution and broth microdilution methods.

MATERIALS AND METHODS

Isorhamnetin

The flavonoid isorhamnetin (3,5,7-Trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-chromen-4-one) was purchased from BLDpharmatech (CAS#: 480-19-3, Shanghai, China). The molecular weight of isorhamnetin is 316.26, and 3'-methylquercetin is the synonym of isorhamnetin. The structural formulation of isorhamnetin is shown in Figure 1.

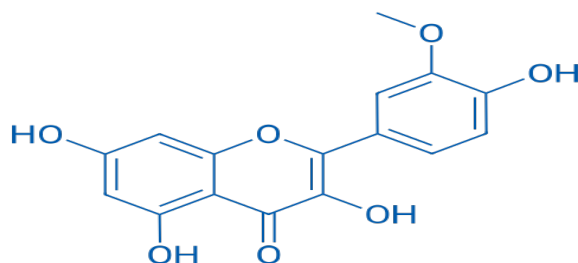


Figure 1. Chemical structure of isorhamnetin (3'-methylquercetin).

Strains and Media

The Minimum Inhibitory Concentration (MIC) values against yeast strains were determined according to the dilution methods (Unver et al., 2023). In this study, 4 yeast strains were used, including *Candida tropicalis* (ATCC 13803), *Candida albicans* (ATCC 14053), *Candida krusei* (ATCC 14243) and *Candida parapsilosis* (ATCC 22019) strains were used. All strains were purchased from the American Type Culture Collection (ATCC). They were subcultured on sabouraud 4 % glucose agar (Chemsolute, Renningen, Germany) and incubated at 35 °C for 24 hours. Subsequently, sabouraud broth (Biolife,

Milan, Italy) and sabouraud glucose agar were used for antifungal microdilution and agar dilution assays, respectively.

Antifungal Assay

Broth Dilution Assay

6 mg of isorhamnetin was dissolved in 100 μ L dimethylsulfoxide (DMSO) (Honeywell, Germany), and 25 μ L of each solution was added to the first well containing 175 μ L of broth. The first well contained 7.5 mg/mL isorhamnetin, with a total volume of 200 μ L. After that, a 100 μ L sample from the first well was added to the second line of the microtiter plate and serially two-fold diluted with sabouraud broth from the 1st to the 10th well. The concentration of isorhamnetin in the wells ranged from 3.75 to 0.007 mg/mL. The standard inoculum was prepared from yeast species, and the density of each species was adjusted to 0.5 McFarland ($1-1.5 \times 10^6$ CFU/mL). 1 μ L of the prepared standard inoculums of *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* were inoculated into A, B, C, and D rows, respectively. The wells in the 11th row were set as a positive control that did not contain isorhamnetin, proving the viability of the microorganism used. The 12th row wells were the negative control, confirming that only broth medium was used and there was no contamination. The microplate was incubated at 35 °C for 24 h. The next day, 10 μ L of resazurin (0.15% w/v) was added to each well to determine the growth of *Candida* species, and the microplate was left in an incubator at 35 °C for 3-4 h. Microorganisms are observed to grow in the wells where blue turns to pink. The experiments were carried out in triplicate, with zero standard deviation.

Agar Dilution Assay

In the agar dilution method, the antifungal activity assay was repeated to confirm the MIC values of isorhamnetin against *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis*. 45 mg of isorhamnetin was dissolved in 1,5 mL DMSO, and this solution was added into 10.5 mL of sabouraud glucose agar at 50 °C. Two-fold dilution was done to obtain an isorhamnetin concentration of 22.5 mg/6 mL in the first agar plate. Therefore, the concentration of isorhamnetin in the agar plates from 1st to 11th ranged from 3.75 to 0.003 mg/mL. After the agar plates were allowed to cool and solidify, they were divided into four areas, and each *Candida* strain was inoculated in these areas separately. A pure sabouraud glucose agar plate was used as a control plate. The standard inoculum of each *Candida* species was prepared in distilled water, and their turbidity was set to 0.5 McFarland. 1 μ L of *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* were inoculated from the standard inoculum onto the agar plates using a blue loop. Subsequently, all plates were incubated at 35°C for 24 h. The next day, the growth of *Candida* species on the plates was evaluated.

Results and Discussion

Results of the Broth Microdilution Method

According to the antifungal activity using the broth microdilution method, MIC values are the values in the well where there is no color change, and the lowest isorhamnetin concentration is used. In Figure 2, it can be seen that the color change begins after the third well. Therefore, the MIC value of isorhamnetin against *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* was found to be 1.875 mg/mL, which is the concentration in the second well, being the same against all microorganisms (Figure 2). All tests were performed in triplicate, and the standard deviation was zero.

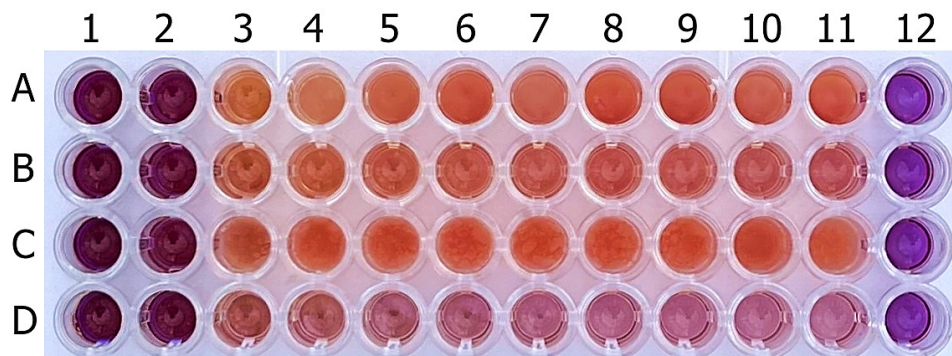


Figure 2. Microplate picture of Isorhamnetin antifungal assay against *C. tropicalis* (A), *C. albicans* (B), *C. krusei* (C), and *C. parapsilosis* (D) using sabouraud broth. 1-10 wells include different concentrations of isorhamnetin (from 3.75 to 0.007 mg/mL) 11. positive control, 12. negative control.

Results of the Agar Dilution Method

The antifungal activity results of isorhamnetin against *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* using agar dilution assay are illustrated in Figure 3. Plates left incubated at 35 °C overnight were evaluated for antifungal activity results. As a result, it was observed that all *Candida* species, including *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis*, showed significant growth on the C plate, where the isorhamnetin concentration was 0.937 mg/mL. No colonies were detected on A and B plates treated with 3.75 and 1.875 mg/mL isorhamnetin, respectively. The MIC is defined as the lowest concentration at which the substance inhibits the microorganisms used in the study, as explained by the Clinical and Laboratory Standards Institute (CLSI, 2018). In this study, the MIC value is described as the lowest concentration of isorhamnetin at which visible growth of the microorganism on the plates is inhibited. Therefore, the concentration (1.875 mg/mL) of isorhamnetin in the B plate was determined as the MIC value for all yeast species (Figure 3). Consequently, the antifungal test results performed with the agar dilution method were the same as the broth microdilution method, supporting the results of the broth microdilution method.

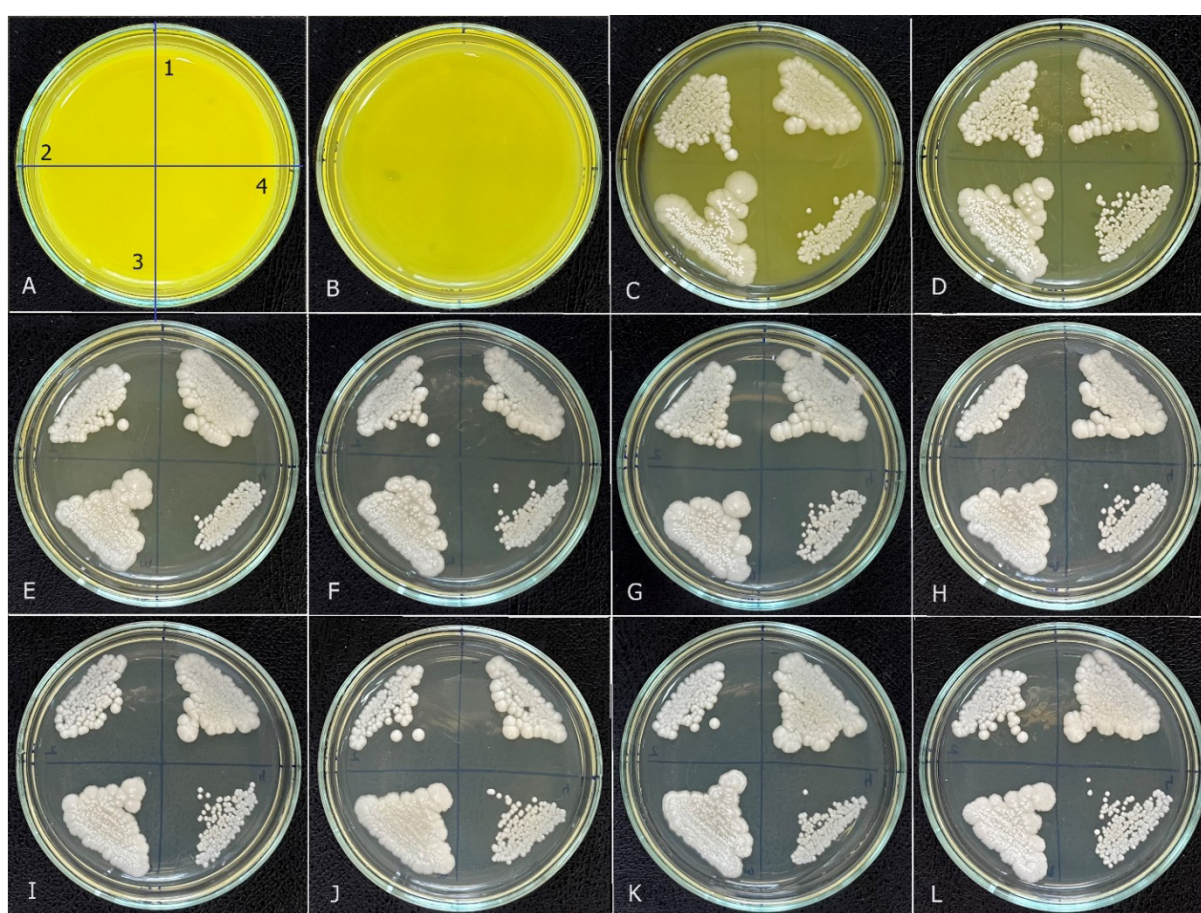


Figure 3. Plate pictures of Isorhamnetin antifungal assay against *C. tropicalis* (1), *C. albicans* (2), *C. krusei* (3), and *C. parapsilosis* (4) using sabouraud glucose agar. Plates from A to K include different concentrations of isorhamnetin (from 3.75 to 0.003 mg/mL). The plate L is positive control.

Flavonoids are natural polyphenolic compounds with two benzene rings flexibly attached to a heterocyclic pyrone ring. Flavonoids show antioxidative activity by inhibiting lipid peroxidation. Therefore, attention has been paid to the protective properties of flavonoids in various plants and foods. (Wattenberg, 1990; Mabry, 1980). Flavonoids constitute a significant portion of the metabolites in plant content and contain more than six thousand nutrients developed in various plants. Numerous studies have been conducted on the antioxidant, anti-allergic, anticancer, antimicrobial, and antiviral properties of flavonoids and their remarkable pharmacological components (Araujo et al., 2014; Li et al., 2014; Kim et al., 2011; Burda & Oleszek, 2001; Ramos, 2007).

Isorhamnetin is the metabolite of quercetin and an important methylated flavonoid due to its pharmacological and therapeutic properties (Khaled, 2020). Isorhamnetin has proven pharmacological effects in many areas. Isorhamnetin has anti-inflammatory and antioxidant properties and may inhibit the growth and metastasis of cancer cells (Wang

et al., 2018; Wei et al., 2018; Chi et al., 2016; Hu et al., 2015; Pengfei et al., 2009). Isorhamnetin plays an essential role in treating diseases through signaling pathways and cytokines. Pharmacologically, isorhamnetin has been evaluated in various studies to have therapeutic effects such as anti-pulmonary fibrosis, anti-osteoporosis, antioxidation, anti-hypoxia, anti-hyperuricemia, regulating immunity, anti-vitiligo and prevention of obesity (Gong et al., 2020; Khaled, 2020). Besides all these, a limited number of antimicrobial studies have been conducted with plant samples containing isorhamnetin. In a study with Kombucha focusing on polyphenolic content, isorhamnetin was associated with changes in bacterial membrane permeability and membrane morphology resulting from intracellular reactive oxygen species (ROS) formation. Isorhamnetin can penetrate the bacterial cell membrane through oxidative stress (Bhattacharya et al., 2018). The antibacterial property of isorhamnetin, which is found in the highest amount (28.79 µg/mg) in *Tamarix ramosissima* bark extract compared to other phenolic compounds, was proven by Ren et al. According to this study, the MIC value of *T. ramosissima* bark extract against bacterial species was found to be 5 mg/mL, and the minimum bactericidal concentration (MBC) value was 10 mg/mL. However, no antifungal activity was observed, and the mechanism of antibacterial activity was not explained (Ren et al., 2019). There appears to be a dilemma between this study and the antifungal study we did. This is because isorhamnetin was not used purely by Ren and his colleagues. Other phenolic compounds found in *T. ramosissima* may also induce the growth of fungal species. It has been stated that the *Ribes nigrum* L. leaf, which contains isorhamnetin in its chemical composition, is a useful antimicrobial, and the isorhamnetin obtained from this extract is bacteriostatic (Stević et al., 2010). In a study where isorhamnetin was found extensively in the plant flower of *Vernonia amygdalina*, the antibacterial activity was attributed to isorhamnetin (Habtamu & Melaku, 2018). However, until today, the studies were conducted with natural substances or plant extracts containing isorhamnetin in their phenolic content. There are very few antimicrobial activity studies with pure isorhamnetin, but these have been studied against a few specific species. The following studies are examples of works with pure isorhamnetin. The antifungal activity of isorhamnetin against *Aspergillus fumigatus*, which causes the serious ocular disease fungal keratitis, was evaluated, and it was observed that isorhamnetin inhibited *A. fumigatus* and reduced inflammatory factors (Tian et al., 2021). Another study using pure isorhamnetin investigated its antituberculosis activity against *Mycobacterium tuberculosis*. This study reported that isorhamnetin reduced IL-1 β , IL-6, IL-12, and INF- γ levels in lung tissue and could be developed as a potent tuberculosis drug (Jnawali et al., 2016).

This study tested the antifungal activity of 97% pure isorhamnetin against *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* using agar dilution and broth microdilution methods. As a result of this study, it was found that isorhamnetin had a strong inhibitory effect against *Candida* species, and its MIC value was 1.875 mg/mL against all *Candida* species. The same study was conducted at the same concentrations against bacterial species: *Staphylococcus aureus*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*. However, no *in vitro* antibacterial effect of 97% pure isorhamnetin was observed at this concentration. Yeast cells are more resistant to antibiotic treatment than bacteria (Memişoğlu, 2019). However, in this study, the antifungal property of isorhamnetin may be attributed to the structural difference of the yeast cells. Consequently, it has been stated that isorhamnetin can be used as a natural therapeutic agent for fungal diseases. This preliminary study shows that isorhamnetin has antifungal activity instead of an antibacterial effect. It also shows that it can be used as an active ingredient against pathogenic opportunistic yeast species without harming the beneficial bacterial prebiotics in the human body. This preliminary study has proven the importance of isorhamnetin, whose pharmaceutical effectiveness has been proven in many medical fields, as a therapeutic or protective agent in infectious diseases.

The limitation of our study is that it was an *in vitro* study, and the effectiveness of isorhamnetin *in vivo* was not tested. Although many *in vitro* studies prove the pharmacotherapeutic properties of isorhamnetin in the literature, *in vivo* studies are limited. Finally, *in vivo* studies are needed to provide more data and clarify the exact mechanism of action of isorhamnetin so that it can be used clinically in treating diseases. The antifungal activity of isorhamnetin has not been tested against any other fungal species belonging to a different genus. However, it has been tested against *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis*, the species most common in fungal diseases, and it was observed that isorhamnetin has a significant inhibitory effect against fungal species. Another limitation of our study is that the inhibitory mechanism of isorhamnetin against *Candida* species has yet to be explained, and a detailed study, perhaps at the molecular level, is needed. However, especially considering the antifungal drug resistance that has occurred in recent years and the severe side effects of synthetic antifungals, proving the antifungal properties of isorhamnetin produces promising results. This preliminary study proved the importance of isorhamnetin as a therapeutic or preventive pharmaceutical agent in infectious diseases.

CONCLUSION

Isorhamnetin is a valuable flavonoid and therapeutic compound with proven healing activity in the medical and pharmacological fields. In the last decade, many studies have been conducted on the anti-osteoporosis, antioxidant, anti-hypoxia, anticancer, and anti-hyperuricemia properties of isorhamnetin. This study is the first to prove the antifungal activity of pure isorhamnetin against *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* (MIC: 1.875 mg/mL). This preliminary study raises the possibility that isorhamnetin can be used as a preventive or therapeutic pharmaceutical agent in infectious *Candida* diseases.

COMPLIANCE WITH ETHICAL STANDARDS

Peer-review

Externally peer-reviewed.

Conflict of interest

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution

Tuba Unver conceived the principal idea, designed and carried out the experiment, wrote the original draft, and took all responsibility for the manuscript.

Ethics committee approval

Ethics committee approval is not required.

Funding

This research was supported by the Research Fund of Inonu University (Project no TSA-2022-3110).

Data availability

Not applicable.

Consent for publication

Not applicable.

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