RESEARCH ARTICLE

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Investigation of the Bioactivity of Hesperidin in an *In Vivo Model of Staphylococcus Aureus* -Induced Osteomyelitis

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Abstract

Osteomyelitis is a severe bone disease that is difficult to treat and causes serious socioeconomic problems. This study aimed to examine the bioactivity of hesperidin in an in vivo *Staphylococcus aureus*-induced osteomyelitis model. Total of 28 male Wistar Albino rats were randomly divided into 4 equal groups (n=7). Groups were designated as Group 1: Control group, Group 2: Sham group, Group 3: Osteomyelitis group, and Group 4: Treatment group (Hesperidin+Osteomyelitis). Unilateral tibial osteomyelitis was induced by administering arachidonic acid and 1×10^6 CFU^{-1} bacterial suspension through a hole drilled from the tibial crest. The rats in the treatment group were given hesperidin once a day by oral gavage for 28 days. At the end of the treatment, the effectiveness of the treatment was evaluated radiographically, biochemically, and histopathologically. The mean scores of intraosseous acute inflammation, intraosseous chronic inflammation, periosteal inflammation, and bone necrosis were evaluated histopathologically. The score was 0 in the control group, 0-2 in the sham group, 9-14 in the osteomyelitis group, and 2-6 in the treatment group. The median values of IAI, ICI, PI, BN, and total histopathological scores of the treatment group were significantly lower than the osteomyelitis group. Biochemically, oxidative stress increased significantly in the osteomyelitis model, however, it significantly decreased in the group treated with hesperidin. Nrf-2 translation levels increased by 0.2% in the sham group compared to the control group and decreased by 26% in the osteomyelitis group but increased by 42% in the treatment group compared to the osteomyelitis group. Compared to the control group, NF-kB translation levels increased by 6% and 21% in the sham and osteomyelitis groups, respectively. However, this value decreased by 9% in the treatment group compared to the osteomyelitis group. Radiographically, the combined score reduced by 65% in the treatment group in comparison to the osteomyelitis group. In conclusion, hesperidin showed anti-inflammatory activity by suppressing NF-kB and antioxidant activity by increasing Nrf-2, both of which play a role in inflammatory pathways. In light of all these findings, it can be said that hesperidin can be used as a potential therapeutic or an agent that can contribute to the treatment of osteomyelitis.

Keywords: Hesperidin, Nrf-2, NF-κB, osteomyelitis, rat.

INTRODUCTION

Osteomyelitis (OM) is a progressive infection that can progress chronically and advance to a permanent stage, resulting in inflammatory destruction, necrosis, and bone deformation. It has been reported that mycobacteria, particularly pyogenic microorganisms, and fungi are also effective in the formation of the disease (Mustafa et al., 2014). Staphylococcus aureus (*S. aureus*), a gram-positive bacterium, is the most commonly isolated bacterium among osteomyelitis infections (Zhang et al., 2021). S. aureus-induced osteomyelitis remains a serious global health problem due to its high recurrence rates and treatment failure. It may not remain solely as a disease but also result in amputation of the affected extremity and even death (Alt and Giannoudis, 2019).

Humans and animals with osteomyelitis present with soft tissue swelling, severe lameness, local pain, and bone lesions. Often there is a history of orthopedic surgery or another bone trauma such as a bite wound. Local lymphadenopathy, fever, depression, and loss of appetite can be observed in the affected cases (May, 2002).

Normal bone tissue is highly resistant to infections. Experimental models usually require a large inoculation of bacteria to induce osteomyelitis. The resulting inflammatory response impairs blood flow and increases intraosseous pressure. This leads to ischemic necrosis. This dead bone tissue, known as the *sequestrum*, can act as a non-living surface for biofilm attachment which allows bacteria to attach. Such a situation makes it difficult for antimicrobial agents and host immune cells to reach the bacteria (Fritz and McDonald, 2008).

Flavonoids are herbal compounds that scavenge hydroxyl, superoxide, alkoxyl, peroxyl, and nitric oxide radicals (Burak and Çimen, 1999). Hesperidin (HSP) was first isolated in 1828 by Lebreton, a French chemist, from the white inner layer of citrus peel (Lebreton, 1828). Compared to other flavonoids, hesperidin stands out with its antioxidant properties (Kuntic et al., 2014). It is abundant in lemon and sweet orange, which are citrus species (Carballo-Villalobos et al., 2016). Pharmacologically, it has anticarcinogenic, antihypertensive (Polat et al., 2016), immunomodulatory (Rezaeyan et al., 2016), anti-inflammatory, strong antioxidative (Banji et al., 2014, Carballo-Villalobos et al., 2016; Yurtal et al., 2020), antiallergenic, neuroprotective, antimicrobial, analgesic and sedative properties (Carballo-

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Villalobos et al., 2016). In the experimental model created in light of all this information, the aim was to investigate the effectiveness of hesperidin, which is known to have high antioxidative and anti-inflammatory properties, for the treatment and prevention of the disease (Yurtal et al., 2020).

The aim of this study is to investigate the therapeutic effect of hesperidin, which has high antioxidant and anti-inflammatory properties, in the chronic osteomyelitis model that is difficult to treat.

MATERIALS AND METHODS

This study was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (dated 30.03.2022 and numbered 2022/03-07). Wistar Albino male rats weighing 250-300 grams were used in the study. The rats were housed at 22 ± 2°C at room temperature, at 12 hours of day/night cycle. The adaptation process of the animals to the environment lasted for 1 week, prior to the start of the experiments, with free access to food and water. Then, the subjects were randomly divided into 4 groups (n=7). The groups were; Group 1: Control group; Group 2: Sham group; Group 3: Osteomyelitis group (OM); Group 4: Treatment Group; Hesperidin (Hesperidin, Sigma-Aldrich, United States) (200 mg/kg, orally, 4 weeks, once daily) (Yurtal et al. 2020) + Osteomyelitis (HSP+OM). To expose the animals to the same stress, rats in groups 1, 2, and 3 were given the same amount of saline (1 ml) as the rats in group 4.

Preparation of the Bacteria Suspension

A colony of S. aureus was inoculated on nutrient agar and incubated at 37°C for 24 hours. A subculture was then grown in the medium for 18 hours at 37°C. This culture was then diluted to obtain 1 x 10^6 colonies/5 microliters.

Infection Induction Procedure

After the subjects were anesthetized intraperitoneally (IP) with a combination of xylazine (Rompun 2%®, 5 mg/kg, Bayer, Turkey) and ketamine (Keta-Control®, 50 mg/kg, Doğa İlaç, Turkey), their right hind legs were shaved and the injection site was cleaned with alcohol. Then, the medullary space was percutaneously entered from the lateral part of the metaphysis of the tibia with an 18-gauge needle (Groups 2, 3, and 4). In group 2, 5 microliters of saline was injected into the medullary canal. Arachidonic acid (Cayman Chemical Company, USA) was used as the sclerosing agent to increase the infection rate (Rissing et al., 1985). First, 5 microliters of arachidonic acid, then 5 microliters (1x10^6) of bacteria suspension (*S. aureus*), and finally 5 microliters of sterile saline Hamilton (Hamilton, USA) were injected into the medullary canal (Groups 3 and 4) (Figure 1). This ensured the entry of the sclerosing agent and all bacteria into the bone marrow (Norden and Kennedy, 1970). The needle was then removed, and the needle hole was sealed with bone wax. Osteomyelitis was evaluated with radiographic, biochemical, and histopathological examinations.

Radiographic Evaluation

The study lasted 4 weeks in total. At the end of the 4th week, the rats were sacrificed under deep anesthesia after taking their intracardiac blood. Radiographs at 45 kilovolts (kV) and 1.3 milliamperes (mAs) (Browiner Beatle-05v, Shenzhen Brownier Technology, China) were taken from the right tibia of the rats in the AP position immediately after the sacrifice.

Radiographic evaluation was performed 4 weeks after inoculation. Periosteal elevation, structural deformation, enlargement of the bone shaft, soft tissue deformation, and new bone formation were assessed for each tibia. Radiographic findings of osteomyelitis were scored between 0 and 4 blindly (0-No sign, 1-Mild, 2-Moderate, 3-Severe, 4-Very severe). The mean scores of each parameter were calculated to obtain a single composite score in each group (Gillaspy et al., 1995).

Histopathological Evaluation

Bones obtained from the experimental animals were fixed in 10% buffered formalin solution for 24 hours. Then, they were taken into 4N formic acid (10%) solution for decalcification. The bones were checked daily until they were decalcified and then washed in running water. Appropriate sections (a transverse section from the proximal tibia and a longitudinal section from the remaining tibia) were taken, and routine follow-up procedures were performed. After the routine follow-up process, sections with 4-5 µm thickness were taken from paraffin-embedded tissue samples and stained with hematoxylin-eosin. The stained sections were examined blindly under binocular light microscopy and osteomyelitis was scored semi-quantitatively according to the predefined criteria (Table 1) (Smeltzer et al., 1997).

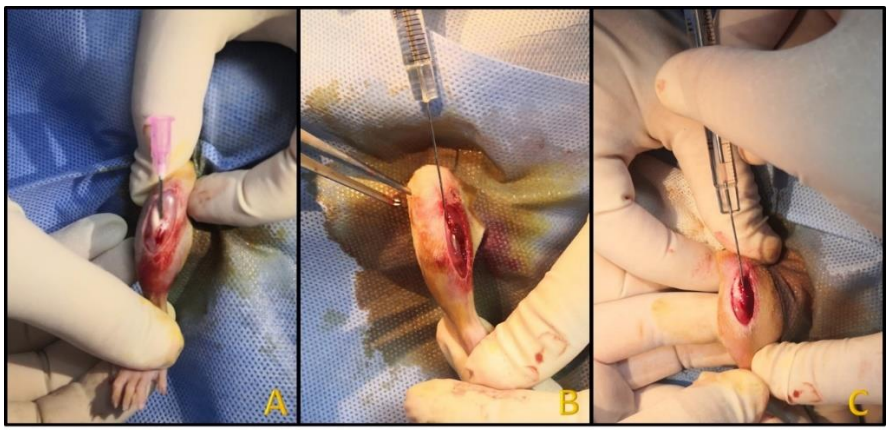

Figure 1. Infection induction procedure a. Entering the medullary canal with an 18 gauge needle tip b-c. Inoculation of the medullary canal with a Hamilton injector.

Table 1. Histological parameters and the scoring system (Smeltzer et al., 1997).

Score	Pathology
	Intraosseous acute inflammation
0	None
1	Minimal to mild inflammation without intramedullary abscess
2	Moderate to severe inflammation without intramedullary abscess
3	Minimal to mild inflammation with intramedullary abscess
4	Moderate to severe inflammation with intramedullary abscess
	Intraosseous chronic inflammation
0	None
1	Minimal to mild chronic inflammation without significant intramedullary fibrosis
2	Moderate to severe chronic inflammation without significant intramedullary fibrosis
3	Minimal to mild chronic inflammation with significant intramedullary fibrosis
4	Moderate to severe chronic inflammation with significant intramedullary fibrosis
	Periosteal inflammation
0	None
1	Minimal to mild inflammation without subperiosteal abscess formation
2	Moderate to severe inflammation without subperiosteal abscess formation
3	Minimal to mild inflammation with subperiosteal abscess formation
4	Moderate to severe inflammation with subperiosteal abscess formation
	Bone necrosis
0	No sign of necrosis
1	Single focus of the sequestrum
2	Multiple sequestrum foci
3	Single focus of necrosis without sequestrum
4	Multiple foci of necrosis without sequestrum

Biochemical Evaluation

After the trial period, surgically collected tissue samples were placed in an ice cuvette and after centrifugation, the supernatants in the tubes were discarded, and the pellet was rinsed three times with ice-cold PBS (1.3 M NaCl, 0.027 M KCl, 0.1 M Na2HPO4 and 0.018 M KH2PO4, pH7.4). Then, 500 µl of lysis buffer (20 mM Tris HCl, pH7.5) containing 1/200 protease inhibitor cocktail (AEBSF, Aprotinin, Bestatin, E-64, Leupeptin, Pepstatin A) was added to each well, and tissue protein samples were obtained. Prepared samples were analyzed with spectrophotometry for TAS (Rel Assay Diagnostics, Turkey) and TOS (Rel Assay Diagnostics, Turkey) analysis, and with ELISA for NF-κB (FineTest, China) and Nrf-2 (FineTest, China) translation levels.

Statistical Analyses

Prior to the significance tests, the Shapiro-Wilk test was performed for normality, one of the parametric test assumptions, and the Levene test was performed for homogeneity of variances. Histopathological scores were expressed as "Median (minimum-maximum)" and radiographic scores as "Mean, standard error, median, minimum and maximum". Biochemical variables were graphically displayed as "mean ± standard error". Since the Shapiro-Wilk test determined that the histopathological and radiographic scores were not normally distributed, nonparametric tests were used. The Kruskal-Wallis test was used for the histopathological and radiographic score differences between the groups, followed by the Mann-Whitney U test with Bonferroni correction for multiple comparisons. The differences in the biochemical variables between the groups were revealed with one-way analysis of variance. Duncan's test was used as a post hoc test for statistically significant variables. Results with $p<0.05$ and below were considered significant. IBM SPSS 22.0 statistical software was used for all statistical analyses.

RESULTS

Histopathological Findings

Intraosseous acute inflammation, intraosseous chronic inflammation, periosteal inflammation, or bone necrosis were not observed in the control group (Figure 2A). Intraosseous acute inflammation (IAI) and bone necrosis (BN) were not observed at all in the sham group, whereas intraosseous chronic inflammation (ICI) and periosteal inflammation (PI) scored a maximum of 1. These findings indicate that the inflammation in the Sham group was negligibly mild, and there was no osteomyelitis (Figure 2B). In the OM group, the IAI score was 3-4, the ICI score was 2-3, the PI score was 3-4, and the BN score was 1-3; and the total score ranged between 9-14 (Figure 2 C-D). In the treatment group, the IAI score was 1-2, the ICI score was 0-2, the PI score was 0-2, the BN score was 0-1, and the total score ranged between 2-6 (Figure 2 E-F).

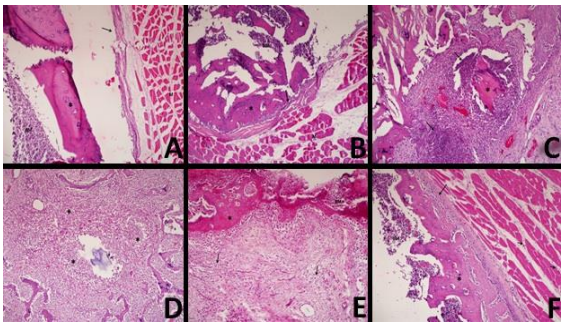

Figure 2. Histopathological Findings (**A**. In the section of the control group, muscle tissue (M), periosteum (arrow sign), bone tissue (asterisk), and bone marrow distance (BM) have normal morphology. There is no inflammation or necrosis. H&Ex100. **B.** In the section of the sham group, muscle tissue (M), periosteum (arrow sign), bone tissue (asterisk), and bone marrow distance (BM) have normal morphology. There is no inflammation or necrosis. H&Ex100. **C.** In the section belonging to the osteomyelitis group, cortical abscess formations (arrows) and bone sequestrum (asterisk) are visible. H&Ex100. **D.** In the section of the osteomyelitis group, widespread active chronic

inflammation and abscess formations (asterisk marks) are observed in the intramedullary area. H&Ex100. **E.** The sections of the treatment group present moderate/severe active chronic inflammation (arrows) without abscess formation. There is no abscess formation or bone necrosis. (Asterisk: bone tissue), BM: bone marrow distance). H&Ex100. **F.** In the sections of another subject belonging to the treatment group, the tissues appear very close to the usual morphology, and there is only mild subperiosteal inflammation (long arrow sign) and mild inflammation in the surrounding muscle tissue (short arrow sign). There is no abscess formation or bone necrosis. (Asterisk: bone, BM: bone marrow distance). H&Ex100).

There were significant differences between the groups in IAI, ICI, PI, BN, and total histopathological score ($p<0.001$, Kruskal Wallis test for all parameters). The histopathological evaluation identified no IAI, ICI, PI, or BN in the control group. In the sham group, IAI and BN were not observed at all, while ICI and PI were only mild. In the OM group, all subjects had intramedullary abscess formation with minimal to severe acute inflammation, mild to severe chronic inflammation, varying degrees of periosteal inflammation, and bone necrosis. Bone necrosis was not observed in only one subject in the OM group. The median total histopathological score of the OM group differed significantly from the Control and Sham groups (both $p<0.001$). These findings confirmed the establishment of the osteomyelitis model. Although IAI and ICI persisted in all subjects in the treatment group, their severity was milder than in the OM group. In the treatment group, 1 subject had mild, the others moderate to severe IAI inflammation; 3 subjects had minimal to mild ICI and 5 subjects had moderate to severe ICI, but none had intramedullary abscess formation or significant fibrosis. Bone necrosis was present in only 2 subjects. The median values of IAI, ICI, PI, BN, and total histopathological scores of the treatment group were significantly lower than the OM group ($p<0.001$, $p=0.005$, $p=0.001$, $p=0.006$, and $p=0.001$; Mann-Whitney U test, respectively) (Table 2). These findings suggest that Hesperidin may contribute to the treatment of osteomyelitis by suppressing inflammation.

Table 2. Statistical data of histopathological findings (Median (min.-max.))

Group	IAI	ICI	PI	BN	Total score
Control	0 (0-0) [c]	0 (0-0) [c]	0 (0-0) [c]	0 (0-0) [b]	0 (0-0) [d]
Sham	0 (0-0) [c]	0 (0-1) [c]	0,5 (0-1) [c]	0 (0-0) [b]	1 (0-2) [c]
OM	3 (3-4) [a]	3 (2-3) [a]	3 (3-4) [a]	2 (0-3) [a]	11 (9-14) [a]
Treatment	2 (1-2) [b]	2 (1-2) [b]	2 (0-2) [b]	0 (0-1) [b]	6 (2-6) [b]

*a,b,c: Different letters in the same column represent statistical significance ($p<0.05$).

Biochemical results

Oxidative Stress Index (OSI=TOS/TAS)

Compared to the control group, an increase of 22% and 54% was observed in the sham group and the OM group, respectively. After osteomyelitis was established, it was found that this value was improved by 58% in the hesperidin group compared to the osteomyelitis group. These findings revealed that oxidative stress increased significantly in the established experimental osteomyelitis model, however, hesperidin used for treatment showed a strong antioxidative effect and significantly reduced oxidative stress (Figure 3A).

Nrf-2

Nrf-2 translation levels increased by 0.2% in the sham (20.94±4.20-$p>0.5$) group compared to the control group, while it decreased by 26% in the OM group (15.38±1.4-$p<0.05$). Yet, this value increased by 42% in the treatment group (22.5±2.4, $p<0.05$) compared to the osteomyelitis group (Figure 3B). These values showed that the decreased Nrf-2 levels, which is the transcription factor of the antioxidative system, were significantly stimulated by hesperidin treatment in the osteomyelitis model.

NF-κB

The NF-kB translation levels increased by 6% ($p>0.05$) and 21% ($p<0.05$) in the sham (3.00±0.53) and OM (3.45±0.4) groups compared to the control (2.83±0.15) group, respectively. Yet, this value decreased by 9% ($p<0.05$) in the treatment group (3.14±0.4) compared to the osteomyelitis group (Figure 3C). These data revealed that the translation levels of the transcription factor NF-kB, which is responsible for the production of inflammation-initiating cytokines, increased significantly in rats with osteomyelitis, whereas, hesperidin administration for treatment significantly decreased the elevated translation levels.

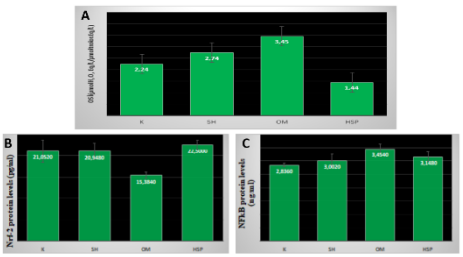

Figure 3: Biochemical analysis results. Figure 3A: OSI values after experiments. Figure 3B: Nrf-2 translation levels. Figure 3C: NF-kB protein levels.

Radiographic Results

Periosteal elevation, structural deformation, enlargement of the bone shaft, soft tissue deformation, and new bone formation were evaluated radiographically (Figure 4). Radiographic findings of osteomyelitis were scored between 0-4 blindly (0-No finding, 1-Mild, 2-Moderate, 3-Severe, 4-Very severe), and statistical data are presented in Table 3. Since only saline was applied in the sham group, the results were statistically similar to the control

group. The OM group had a significantly higher composite score than the sham and control groups ($p<0.05$). The comparison of the OM group with the treatment group showed that, although not statistically significant, hesperidin provided some improvement that could be clinically significant.

Table 3. Radiographic scoring of rats in the experimental groups

Variable	Group	Arith. Mean	Std. Error	Median	Minimum	Maximum	p	Letter *
Total Score	Control	0.00	0.00	0.00	0.00	0.00	<0.001	c
	Sham	2.29	0.42	3.00	0.00	3.00		bc
	OM	9.57	0.78	9.00	7.00	12.00		a
	Treatment	6.29	0.78	7.00	3.00	8.00		ab

*a,b,c: Different letters in the same column represent statistical significance ($p<0.05$).

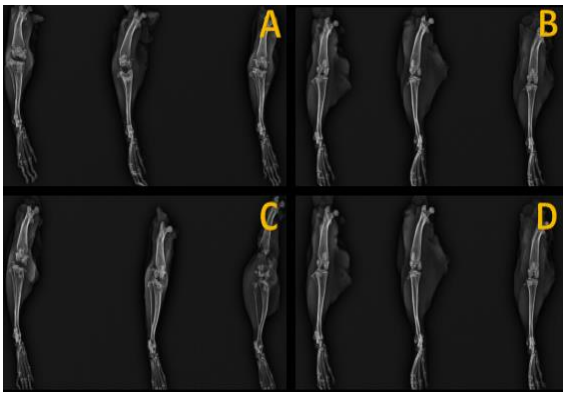

Figure 4. Radiographic findings A: No pathological findings in the radiographic images of the control group. B: Mild inflammatory changes in the proximal tibia in the radiographic images of the Sham group. C: Severe bone deformations and sequestrums in the proximal tibia in the OM group. D: Radiographic findings are milder and bone deformations are less common in the treatment group than in the OM group.

DISCUSSION AND CONCLUSION

The treatment of osteomyelitis, which is one of the musculoskeletal system infections, creates a significant burden on both the physician and the health system (Birt et al., 2017). Treatment of osteomyelitis requires drainage, extensive debridement, obliteration of the dead space, wound care, stabilization of the bone (if necessary), and the use of culture-directed antibiotics (Carek et al., 2001; Calhoun et al., 2009). Yet, osteomyelitis is a disease known to be persistent and resistant to conventional antibiotic therapy due to the low penetration of antibiotics into the infection site (Kadry et al., 2004). The aim of this study is to develop a new supportive treatment regimen using hesperidin, whose high antioxidative, anti-inflammatory, and antimicrobial properties have been proven in a rat model of chronic staphylococcal osteomyelitis.

In hematogenous osteomyelitis, a single pathogen is almost always isolated from the bone. In adults, the most commonly isolated organism is Staphylococcus aureus (Carek et al., 2001; Lew and Waldvogel, 2004; Calhoun et al., 2009) and the incidence of the disease is higher in men than in women (Roesgen et al., 1989; Massaccesi et al., 2022). Therefore, the present study used S. aureus to model osteomyelitis in male rats.

The rat tibial osteomyelitis model was well-described by Rissing et al., (1985). Sclerosing agents such as sodium morrhuate or arachidonic acid were used to create a progressive osteomyelitis model and increase bacterial uptake (Rissing et al., 1985; Mendel et al., 2005). This model establishes progressive osteomyelitis and histopathologically resembles human chronic osteomyelitis. It has also been widely used since it was developed (Rissing et al., 1985; Gratz et al., 2001; Mendel et al., 2004; An et al., 2006). In the present study Arachidonic acid was also used as the sclerosing agent.

It is known that hesperidin has anti-inflammatory, strong antioxidative, and antimicrobial activity (Yurtal et al., 2020). Kostic et al., (2020) tried phenolic compounds such as caffeic acid, chlorogenic acid, ferulic acid, morin, quercetin, isoquercitrin, rutin, and hesperidin in their in vitro S. aureus study. All phenolic compounds inhibited bacterial uptake by more than 60% in clinical isolates. Among the antibiotics used in the study, only Hesperidin showed a strong antimicrobial effect by inhibiting the uptake of S. aureus ATCC 11632, defined as the standard strain, by over 60% (Kostić et al., 2020). In the present study, the antimicrobial activity of Hesperidin was investigated as in vivo on live animals. This is the first in vivo study to investigate the therapeutic effect of Hesperidin on osteomyelitis.

The research revealed that hesperidin exerted its anti-inflammatory effect by decreasing NF-κB protein levels and its antioxidative effect by increasing NrF-2 protein levels compared to the osteomyelitis group. NF-κB, a nuclear transcription factor, is involved in the inflammatory, immune, and stress responses. NF-kB contributes to both innate and adaptive immunity by regulating the production of proinflammatory cytokines (Li and Verma, 2002). Activation of NF-kB in cells results in increased production of proinflammatory mediators such as IL-1, IL-6, interleukin 8 (IL-8), and TNF. Sustained inhibition of NF-kB activity has proven effective in controlling inflammatory diseases in several animal models. For example, the blockage of NF-kB activity has been shown to prevent both inflammation and tissue damage in rheumatoid synovium (Bondeson et al., 1999).

The effects of hesperidin on NF-kB signaling have been documented using mouse models. Xiao et al. (2018) reported that the underlying cause of the anti-inflammatory activity of Hesperidin is the blockage of the NF-kB pathway. A mouse model of diabetes has shown that hesperidin reduces the NF-kB levels (Iskender et al., 2017). Pinho Ribeiro et al., (2015) reported that hesperidin suppressed the activity of NF-κB and showed anti-inflammatory activity in a mouse model of pain (Pinho Ribeiro et al., 2015). Guazelli et al., (2021) reported that hesperidin methyl chalcone showed anti-inflammatory activity by inhibiting TNF-alpha, IL-1B, and NF-kB activation in experimental ulcerative colitis.

Polyphenols, one of the most common flavonoids, are widely found in plants. Hesperidin is a flavanone glycoside and is abundant in citruses. HSP has many beneficial medicinal effects thanks to its antioxidative,

anti-inflammatory, anti-carcinogenic, antiallergic, and neuropharmacological properties (Li and Schluesener, 2017). Many rodent models have shown that it exerts anti-inflammatory activity by suppressing cytokine release and oxidative stress and reducing NF-kB activation (Pinho-Ribeiro et al., 2015). Recent studies have shown that hesperidin and hesperetin have a protective effect on tissues due to their effects against oxidative stress caused by various oxidants such as peroxynitrite and hydrogen peroxide and some damaging chemicals and toxins (Kalpana et al., 2009; Kamaraj et al., 2010; Shrivastava et al., 2013; Kawaguchi et al., 2004; Kim et al., 2004). Kalpana et al., (2009) investigated different aspects of the antioxidative and protective effects of Hesperidin on membrane damage caused by H_2O_2 in red blood cells. They have shown that they protect cell membranes and oxidative stress-induced DNA breaks by enabling oxidative molecules such as H_2O_2 to aggregate.

Numerous studies have shown that HSP neutralizes reactive oxygen species (ROS) such as superoxide anions and hydroxyl radicals, and reactive nitrogen species (RNS) such as peroxynitrite and nitric oxide radicals. (Garg et al., 2001; Kim et al., 2004; Wilmsen et al., 2005). Guazelli et al., (2021) determined that hesperidin showed antioxidative activity by suppressing myeloperoxidase activation and increasing the amount of glutathione. In an LPS-induced RAW cell inflammation model, Kang et al., (2011) demonstrated that the flavonoid mixture (nobiletin, Naringin, and HSP) including hesperidin showed anti-inflammatory activity by significantly reducing the levels of iNOS and COX-2 messenger ribonucleic acid (mRNA), and especially by suppressing NF-κB and mitogen-activated protein kinases (MAPK) signaling pathways. Nrf2 activation is a crucial mechanism in defense against oxidative stress. Nrf2 activates the expression of genes coding antioxidative and cytodefensive proteins involved in the detoxification and elimination of electrophilic agents and reactive oxidants (Nguyen et al., 2009).

Xin et al., (2020) found that hesperidin significantly increased the mRNA and protein levels of Nrf-2 in the hypobaric hypoxia model compared to the hypoxia group. However, the researchers also reported that hesperidin exerts its beneficial effect by stimulating the Nrf-2/HO1 antioxidant signaling pathway. Again, Aly et al. (2017) showed that in diethyl nitrosamine/carbon tetrachloride-induced renal damage, hesperidin protects cells by increasing the expression levels of genes in the Nrf-2/HO1 antioxidative signaling pathway. In an oxidative stress-related model of aging, Elavarasan et al., (2012) reported that hesperidin protects cells with antioxidant capacity stimulation by increasing catalase and superoxide dismutase enzyme activities, especially due to the increase in Nrf-2 protein level in heart tissue. Jain and Parmar, (2011) showed that hesperidin suppressed lipid peroxidation, increased superoxide dismutase, and catalase activity, and significantly decreased reduced glutathione and nitric oxide in the tissue in a rat air bladder inflammation model. In this study, hesperidin showed antioxidant activity by decreasing the OSI value and increasing the Nrf-2 value in the treatment group.

Most studies in the literature have also focused the longitudinal axis of the tibia (Smeltzer et al., 1997, Fukisima et al., 2005, Güzel et al., 2016). In a tibial osteomyelitis model, Chadha et al., (1999) reported that locations close to the growth plate of the tibia were infected and sections were taken from the proximal tibia. Therefore, in our study, to evaluate the proximal tibia in two axes, the tibia was sampled longitudinally after taking a transverse section from the proximal tibia. The histopathological diagnosis of osteomyelitis includes the evaluation of many parameters such as intramedullary acute inflammation, intramedullary abscess formations, intraosseous chronic inflammation, fibrosis, periosteal reaction, bone necrosis, and sequestered bones (Sybenga et al., 2019). This study used the histopathological scoring of Smeltzer et al., in 1997, which evaluated these parameters together and presented the scoring with objective criteria. Accordingly, intraosseous acute inflammation, intramedullary abscess formations, intraosseous chronic inflammation, fibrosis, periosteal inflammation or bone necrosis were evaluated and scored for each subject. The histopathological examination revealed no IAI, ICI, PI, or BN in the control group, while inflammation was negligibly mild in the Sham group. In the OM group, all subjects had intramedullary abscess formation with minimal to severe acute inflammation, mild to severe chronic inflammation, varying degrees of periosteal inflammation, and bone necrosis. All subjects in the OM group, except one, had a variable rate of bone necrosis. The significant difference between the total histopathological score of the OM group and the total scores of the Control and Sham groups confirmed that the osteomyelitis model was established. Although IAI and ICI persisted in all subjects in the treatment group, their severity was milder than in the OM group. In the treatment group, 1 subject had mild, the others moderate to severe IAI inflammation; 3 subjects had minimal to mild ICI and 5 subjects had moderate to severe ICI, but none had intramedullary abscess formation or significant fibrosis. Bone necrosis was present in only 2 subjects. The statistically significantly lower IAI, ICI, PI, BN, and total histopathological scores of the treatment group compared to the scores of the OM group suggests that Hesperidin can contribute to the treatment of osteomyelitis by suppressing inflammation.

The signs of osteomyelitis were clearly demonstrated by biochemical, histopathological, and radiographic changes. Although in vitro studies (Kostić et al., 2020) have been conducted on the use of Hesperidin in osteomyelitis, there is no in vivo study in the literature. To the best of our knowledge, this is the first in vivo study to investigate the therapeutic effect of Hesperidin on osteomyelitis.

As a result, hesperidin histopathologically decreased IAI, ICI, PI and BN in the treatment group significantly compared to the osteomyelitis group. Biochemically, it showed a visible anti-inflammatory activity by suppressing NF-kB, which is involved in inflammatory pathways, and increasing Nrf-2 in the treatment group. Although it was not statistically significant in the radiological treatment group compared to the osteomyelitis group, it showed a clinically significant improvement. It can be added to the standard treatment protocol to treat the chronic course of osteomyelitis and to break the resistance of the disease to conventional therapy. This study is the first in vivo study investigating the therapeutic effect of hesperidin on osteomyelitis in the literature, and our findings may guide future experimental and clinical studies on the treatment of osteomyelitis.

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Conflict of Interest
The authors declare that they have no competing interests.

Authorship contributions
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