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Antimicrobial Effects of Chitosan Extracted from Crayfish Shells in Cream Formulations

Kerevit Kabuklarından Elde Edilen Kitosanın Krem Formülasyonlarındaki Antimikrobiyal Etkileri

Türk Denizcilik ve Deniz Bilimleri Dergisi

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ABSTRACT

The objective of the present study was to investigate the antimicrobial properties of a chitosan-based cream. To achieve this, the antimicrobial effects of a cream enriched with chitosan were compared with those of a control group. Chitosan, sourced from crayfish obtained frozen from Eğirdir Lake, Eğirdir, Isparta, served as the primary material. The study involved a comparison between control (F1) and treatment (F2) groups. While both cream formulations exhibited bacterial inhibition, only the F1 formulation demonstrated a significant reduction in viable microorganisms for C. albicans. The cytotoxicity assessment revealed a concentration-dependent increase in the cytotoxic effects of the samples. Notably, the F1 formulation exhibited higher toxicity to healthy cells compared to the F2 formulation. In conclusion, further investigation is necessary to understand the mechanisms underlying their cytotoxic effects and to optimize their formulations to enhance biocompatibility. Moreover, the chitosan-based cream developed in this study demonstrated notable antimicrobial efficacy against the tested bacteria.

Keywords: Chitosan, Crayfish, Antimicrobial effect, Cytotoxicity, Skin health

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ÖZET

Bu çalışmanın amacı, kitosan bazlı bir kremin antimikrobiyal özelliklerinin araştırılmasıdır. Bunun için, kitosan ilave edilmiş bir kremin antimikrobiyal etkileri kontrol grubu ile karşılaştırılmıştır. Ana materyal olarak, çalışma kapsamında Eğirdir Gölü'nden dondurulmuş bir şekilde temin edilen kerevitlerden ekstrakte edilen kitosan kullanılmıştır. Çalışma, kontrol (F1) ve muamele (F2) grupları arasında bir karşılaştırmayı içermektedir. Her iki krem formülasyonu da bakteriyel inhibisyon sergilerken, yalnızca F1 formülasyonu C. albicans için canlı mikroorganizmalarda önemli bir azalma göstermiştir. Sitotoksisite değerlendirmesi, örneklerin sitotoksik etkilerinde konsantrasyona bağlı bir artış olduğunu ortaya koymuştur. Özellikle, F1 formülasyonunun sağlıklı hücreler üzerinde F2 formülasyonuna kıyasla daha yüksek bir toksisite sergilediği gözlenmiştir. Sonuç olarak, sitotoksik etkilerinin altında yatan mekanizmaları anlamak ve formülasyonlarını biyouyumluluğu artıracak şekilde optimize etmek için daha fazla araştırma yapılması gerekmektedir. Ayrıca, bu çalışmada geliştirilen kitosan bazlı krem, test edilen bakterilere karşı dikkate değer antimikrobiyal etkinlik göstermiştir.

Anahtar sözcükler: Kitosan, Kerevit, Antimikrobiyal etki, Sitotoksisite, Deri sağlığı

1. INTRODUCTION

The rapid expansion of the global population necessitates the more efficient utilization of limited food resources. Consequently, the aquaculture industry has garnered significant attention on a global scale, aligning with the escalating population growth, and has substantially bolstered the overall production of animal protein (Gomez et al., 2019; Bohnes et al., 2020; Regueiro et al., 2021). In this context, the sector of food production, particularly animal protein production, plays an important role in nutrition and food safety (Maulu *et al.,* 2022). The seafood production sector is thought to be growing rapidly and demonstrating enhanced environmental performance compared to land-farmed animals (Troell *et al.,* 2019).

Seafood encompasses significant animal protein sources obtained through aquaculture and fishing. These products differ from land-based protein sources in that they contain essential micronutrients and fatty acids important for overall health (FAO, 2022). Additionally, shellfish and other seafood products contribute to ecologically advantageous nutrient cycles, making them environmentally beneficial systems. Therefore, they can be a crucial resource for sustainable food production in the future (Cai *et al.,* 2019).

Global seafood production recorded a growth of 69.2% from 2002 to 2022, reaching 184.6 million tons in 2022. It is reported that more than half of production is obtained from aquaculture (FAO, 2022). Shellfish account for more than 9% of total seafood production (FAO, 2022). Shellfish, especially species such as shrimp, crab, and lobster, constitute a diverse group of arthropods and play a significant role in aquaculture. Observations indicate that the shells of these crustaceans, especially lobster shells, hold potential for biological use and inspire new research topics (Chen *et al.,* 2020; Mazlum *et al.,* 2022; Mazlum and Yazıcı, 2023).

Crayfish, which play a pivotal role in freshwater ecosystems, are known to consume a diverse array of resources including other invertebrates, macrophytes, algae, and detritus. Moreover, they demonstrate cannibalistic behavior and display selective feeding habits, preferring certain invertebrates and macrophytes (Mazlum *et al.*, 2021). Crayfish, a popular freshwater organism, contributes directly to the food chain and species diversity when consumed in large quantities. Crayfish are preyed upon by numerous predators, thereby serving as a vital energy source within upper trophic levels. From an economic perspective, crayfish represent a valuable food resource (Nyström, 2002).

Freshwater crayfish, classified within the phylum Arthropoda, class Crustacea, and order Decapoda, comprise a total of over 737 species (Crandall and De Grave, 2017). Crayfish, widely consumed worldwide and recommended as a

high-quality food source as part of a balanced diet, have contributed to their growth in the market, establishing them as a valuable resource for human nutrition. Crayfish shells, considered by-products produced in approximately 100.000 tons annually, vary based on species, season, and habitat characteristics (Peng *et al.*, 2016). During crayfish processing, the waste ratio typically falls between 40% and 50%, with chitin comprising 40% of these by-products (Cai *et al.*, 2017). Crayfish shells are rich in omega-3 fatty acids, pigments, 20–30% protein, 30–40% calcium carbonate, and 20–30% chitin. The commercial value and unique properties of these shells have garnered increased attention, leading researchers to explore various applications (Cai *et al.*, 2017). Crayfish exoskeletons are believed to meet emerging research criteria including biomaterial structure, diversity, sustainability, function, and cost-effectiveness (Binnewerg *et al.*, 2020; Araujo *et al.*, 2021; Nekvapil *et al.*, 2021). These properties make crayfish shells valuable in various industrial applications and attract the attention of researchers (Cai *et al.*, 2017). Chitin, a polysaccharide found in crayfish shells, is one of the most abundant natural biopolymers in nature. Previous studies have demonstrated the potential of chitin to be transformed into chitosan, a higher value-added product used in various sectors.

Due to safety and comfort during use, chitosan has replaced synthetic polymers in ophthalmic applications (Dutta et al., 2012). Lenses derived from chitosan offer benefits for ocular injuries owing to their antimicrobial and wound healing attributes (Figiela et al., 2018). Moreover, chitosan has found application as a seed coating agent aimed at pest management and enhancement of plant resilience against microorganisms (Zeng et al., 2010). It has been reported that chitosan affects the growth and germination of Ajowan (Trachyspermum ammi) seeds (Mahdavi and Rahimi, 2013).

Chitosan and its derivatives are preferred due to their plant protective properties against fungi, viruses, bacterial diseases and nematodes (Ramírez *et al.*, 2010). As a wound dressing, chitosan can promote faster regeneration of skin epithelial cells and collagen production by fibroblasts. Adding chitosan to food prevents microbial growth, unpleasant appearances, bad tastes and economic losses. Studies have shown that adding chitosan to cheese improves its microbiological quality, inhibits mold and yeast growth, and extends its shelf life (El-Diasty *et al.*, 2012). The antibacterial properties of chitosan also make it suitable for use as an edible active packaging material (Muzzarelli and Muzzarelli, 2005). Chitosan has been shown to increase fertilizer absorption, release nitrogen, and act as a bio stimulant to increase crop yields. Chitin and chitosan find applications in food, pharmaceuticals, cosmetics, textiles and various industrial sectors. Moreover, its attractive properties such as biodegradability, biocompatibility, biodegradability, and non-toxicity contribute to its importance in providing health benefits (Casadidio *et al.*, 2019).

The purpose of current study was to reveal the effects of antimicrobial properties of chitosan-based cream. For this purpose, the antimicrobial effects of chitosan added cream were compared with the control group.

2. MATERIALS AND METHODS

2.1. Chitosan source and extraction

Crayfish were obtained frozen from Eğirdir Lake, Eğirdir, Isparta (Figure 1).

Figure 1. Crayfish shell materials used in chitosan extraction

Crayfish shells were used as the primary source for chitin extraction. Chemical and biological methods are commonly used to extract chitin from these shells (Pachapur *et al.*, 2016).

The crayfish shells were first washed, dried and ground (Figure 2).

Figure 2. A view of ground crayfish (*Pontastacus leptodactylus*) shells

For chitin extraction, demineralization, and decoloration processes were applied respectively (Pal *et al.*, 2014). Chitosan used in the study was obtained after deacetylation (strong alkaline treatment with 12.5 M NaOH). Before using chitosan in the cream formulation, it was prepared with glacial acetic acid at the desired concentration (Trung *et al.*, 2020) (Figure 3).

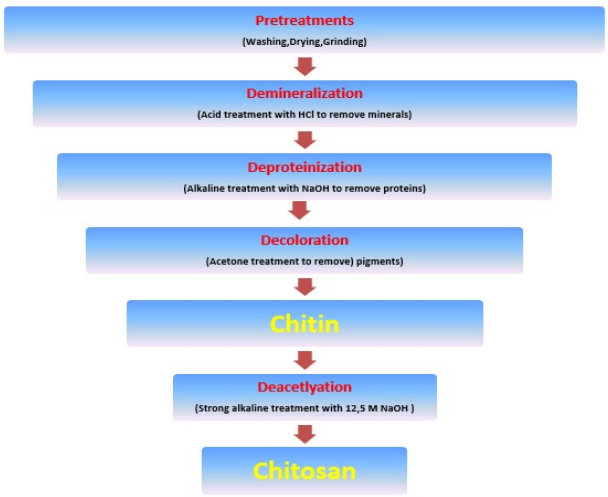

Figure 3. Schematic representation of chitin and chitosan production

2.2. Preparation of chitosan-based cream

Chitosan-based cream formulations are given in Table 1.

Table 1. Chitosan-based cream formulations

Phases	Bileşen	F1(%)	F2(%)
A	Gum	0.3	0.3
A	Glycerine	4	4
A	Distilled Water	69.2	65.7
B	Emulsifier (OLIVEM 1000)	6	6
B	Cetyl Alcohol-Cetyl Alcohol (Vegetal Derived Thickener) -Nafol50/50	2	2
B	Jojoba oil	7	7
B	Argan oil	6	6
B	Shea butter	3	3
C	Vitamin E	0.5	0.5
C	Natural Herbal Preservative (Lexgrad Natural)	1.5	-
C	Chitosan	-	5
C	Lavender Essential Oil	0.5	0.5
	Total	100	100

A, B and C represent phases of cream production

2.3. Microorganisms

In this study, microbial strains *Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 25923 and Candida albicans ATCC 10231* were used for the challenge test. The bacterial strains were activated on Tryptic Soy Agar (TSA) (Merck, Germany) at 37°C for 24 hours while the fungal strain was activated on Sabouraud dextrose agar (SDA) at 30°C for 48 hours. Activated microorganisms were suspended in a physiological saline solution to obtain a density equal to 0.5 McFarland turbidity (approximately 1.5 X 10^8 CFU/mL).

2.4. Challenge test

The microbial control test was conducted following the procedures outlined in the European Pharmacopoeia (EP) guideline 7.0, 2010. Twenty grams of the cream samples (F1 and F2) were aseptically transferred into sterile petri dishes and subsequently inoculated separately with 0.2 mL of bacterial suspension

and 0.1 mL of fungal suspension. The inoculated samples were thoroughly mixed and then incubated in the dark at 20–25°C. On days 0, 7, 14, and 28 post-inoculation, 1 g of the inoculated samples was aseptically withdrawn, diluted in physiological saline, and spread onto TSA agar plates for bacterial analysis and SDA agar plates for fungal analysis. The plates were then respectively incubated at 37°C and 28°C for bacteria and fungi. Following the incubation period, the number of viable cells in the samples was enumerated. The results were reported as log colony-forming units per milliliter (log CFU/mL). Each experiment was conducted in triplicate (Fang *et al.*, 2016).

2.5. Cytotoxicity

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay was used to analyze the cytotoxic activities of cream samples F1 and F2 against the L929 mouse fibroblast cell line (ISO 10993-5). The 96-well cell culture plates were seeded with $1x10^4$ cells per well and incubated for 24 h. The cream samples were applied to cells in eight different concentrations (1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.007813 mg/mL). The cell culture plates were incubated in the incubator for 24 hours at 37°C and 5% CO_2.The samples were applied three times at each of the concentrations tested. DMEM medium without samples was used as the negative control. Following the incubation period, 50 µL of MTT solution (1 mg/mL) was added to each well. After 2 hours of further incubation at 37°C, 100 µL of isopropanol was added to the wells, and the absorbance values were measured at 570 nm using a microplate reader to determine cell viability. The percent viability was calculated relative to the control groups, employing the following formula:

$$\text{Cell Viability}\,(\%) = \frac{\text{Sample OD}}{\text{Control OD}} \times 100 \qquad (1)$$

The cytotoxicity studies were made in triplicate and the data were given as mean ± standard deviation (SD). A paired samples t-test was used to compare the control (F1) and treatment (F2) groups.

3. RESULTS

Figure 4 and Figure 5 show chitin and chitosan obtained from crayfish (*Pontastacus leptodactylus)* shell, respectively.

Figure 4. Chitin obtained from crayfish (*Pontastacus leptodactylus*) shell (Mazlum *et al.*, 2022)

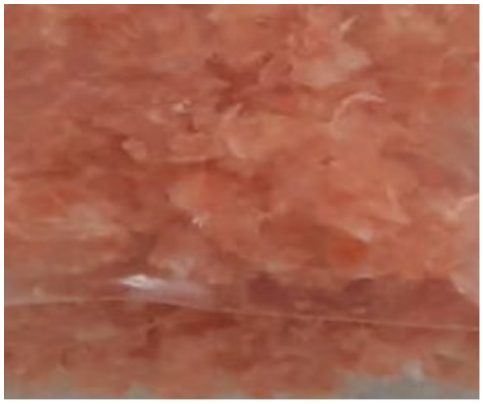

Figure 5. Chitosan obtained from crayfish (*Pontastacus leptodactylus*) shell (Mazlum *et al.*, 2022)

According to the European Pharmacopoeia (EP), "The preservative properties of the preparation are considered adequate if, under the conditions of the test, there is a significant decrease or no increase, as appropriate, in the number of microorganisms in the inoculated preparation after the specified durations and at the prescribed temperatures". The criteria for evaluation of antimicrobial activity are a minimum 3 log reduction after 7 days of inoculation for bacteria, a minimum 2 log reduction after 14 days of

inoculation for fungi, and no increase in number of viable microorganisms at 28 days compared to the previous reading. Growth inhibition of tested microorganisms in cream formulations are presented in Figure 6. The log reduction in the number of viable microorganisms is given in Table 2.

Although both cream formulations met the criteria for bacterial inhibition, only the F1 formulation caused the necessary reduction of viable microorganisms for *C. albicans*.

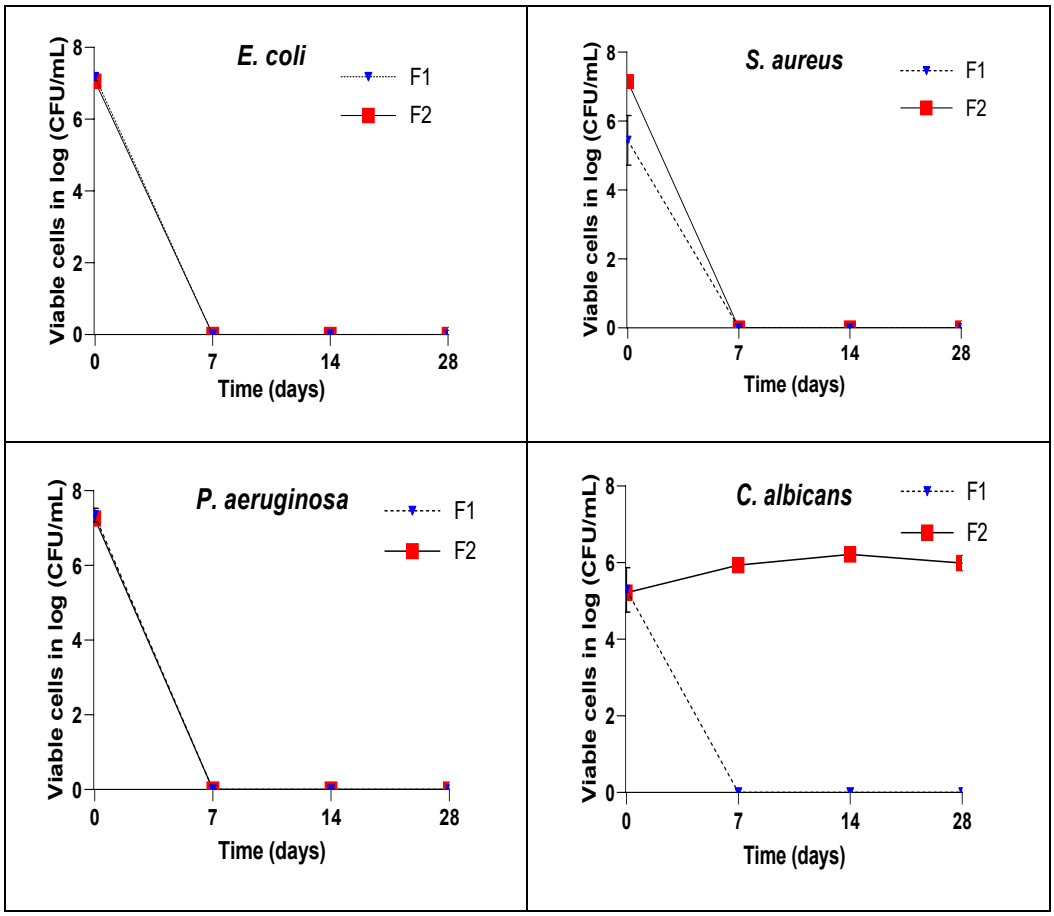

Figure 6. Growth inhibition of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231 in cream formulations F1 and F2.

Table 2. The log reduction in the number of viable microorganisms

	F1			F2		
	7 d	14 d	28 d	7 d	14 d	28 d
Escherichia coli	>3	>3	>3	>3	>3	>3
Staphylococcus aureus	>3	>3	>3	>3	>3	>3
Pseudomonas aeruginosa	>3	>3	>3	>3	>3	>3
Candida albicans	>2	>2	>2	NR*	NR	NR

*Not reduction

Upon analysis of the cytotoxic activity results, it was observed that the samples exhibited increased cytotoxic effects in a concentration-dependent manner (Figure 7). The toxicity of the F1 formulation on healthy cells was found to be higher than that of the F2 formulation.

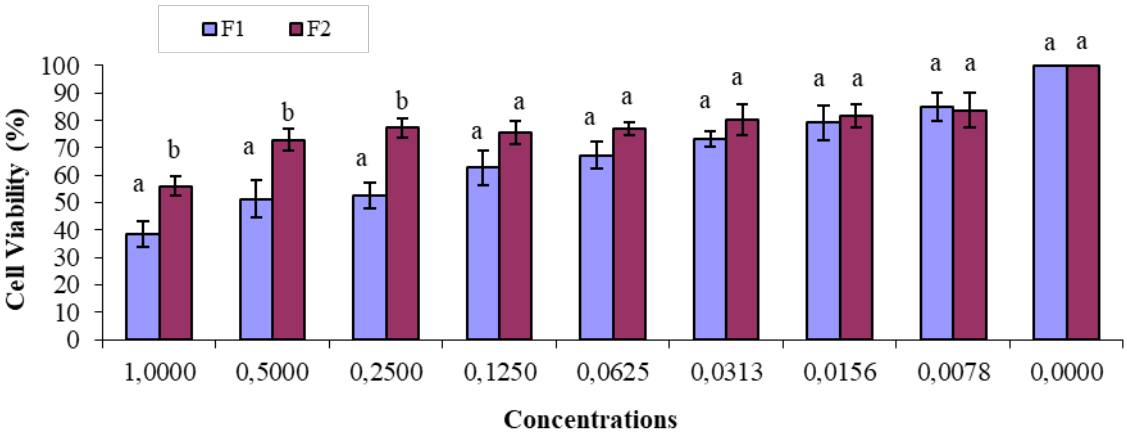

Figure 7. The cytotoxic activities of cream samples against L929 mouse fibroblast cell line

4. DISCUSSIONS

The current study aimed to investigate the antimicrobial effects of adding chitosan extracted from crayfish shells to the control group formulation. Within the scope of the study, control (F1) and treatment (F2) groups were compared. There are studies in the literature demonstrating the beneficial effects of cosmetic products containing chitosan on wound healing (Kim *et al.*, 2015; Ahmad *et al.*, 2018; Bektas *et al.*, 2020). Studies in which chitosan is added to cosmetic products in different forms such as nanoparticles are also available in the literature (Ta *et al.*, 2021; Mondéjar-López *et al.*, 2022a, b). In addition, there are also studies in which different plant extracts are added to the formulations to increase the antimicrobial effect of chitosan in the cream formulation (Wisuitiprot *et al.*, 2022; Parwati and Wikantyasning, 2023). Pérez-Díaz *et al.* in 2016 showed that cell viability in human fibroblasts changed depending on the concentration in chitosan gel formulations to which AgNPs were added. These results support that chitosan gel formulations loaded with AgNPs show good biocompatibility. The current study supports the literature information that the chitosan-containing cream formulation has lower cytotoxicity on fibroblasts. However, different natural resources can also be utilized to improve the biocompatibility of these chitosan-containing cosmetic products (Azuma *et al.*, 2015; Movaffagh *et al.*, 2022; Bagheri *et al.*, 2022). Khattaket *et al.* (2022) compared the antimicrobial effect of the cream prepared by adding chitosan with the antimicrobial effect of the cream containing only bacitracin. Researchers found that the cream containing chitosan had similar antimicrobial effects on *E. coli*, *S. aureus*, *B. cereus* and *P. aeruginosa*. Chaiwong *et al.* (2022) evaluated the antimicrobial effect of Mangosteen extract deodorant cream to which they added carboxymethyl chitosan synthesized from high molecular weight natural chitosan.

5. CONCLUSIONS

In conclusion, the results of the study revealed that the tested cream had an antimicrobial effect on *S. aureus*, *E. coli* and *P. aeruginosa* bacteria. Chitosan-based creams have received considerable attention for their potential biomedical applications, such as wound healing and skincare. It is essential to understand the cytotoxicity of these creams to evaluate their safety and effectiveness. Further research is necessary to clarify the mechanisms underlying their cytotoxic effects and to optimize their

formulations for improved biocompatibility. Although it was observed that the chitosan-based cream produced in this study had a good antimicrobial effect on the bacteria studied, it is suggested that some plant extracts can be used to increase the effect of this formulation on yeasts.

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AUTHORSHIP CONTRIBUTION STATEMENT

Yavuz MAZLUM: Conceptualization, Validation, Resources, Writing - Original Draft, Writing-Review and Editing, Data Curation, Software, Visualization, Supervision, Project administration. **Selin SAYIN:** Conceptualization, Methodology, Validation, Formal Analysis, Resources, Writing - Original Draft, Data Curation, Software, Visualization, Supervision, Project administration. **Betül AYDIN:** Methodology, Validation, Formal Analysis, Resources, Writing - Original Draft, Writing-Review and Editing, Data Curation, Software, Visualization, Supervision. **Mehmet NAZ:** Methodology, Validation, Formal Analysis, Resources, Writing - Original Draft, Writing-Review and Editing, Data Curation, Software, Visualization, Supervision. **Metin YAZICI:** Conceptualization, Methodology, Validation, Formal Analysis, Resources, Writing - Original Draft, Writing-Review and Editing, Data Curation, Visualization, Supervision. **Şerife AKKÜÇÜK:** Methodology, Validation, Formal Analysis, Resources, Writing-Review and Editing, Data Curation, Visualization, Supervision, Project administration

CONFLICT OF INTERESTS

The author(s) declare that for this article they have no actual, potential or perceived conflict of interests.

ETHICS COMMITTEE PERMISSION

No ethics committee permissions is required for this study.

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