

Determination of essential oil composition of *Laurencia obtusa* and *Ceramium* spp. in Black Sea and antileishmanial activity

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Abstract: In this study, the chemical compositions of volatile oils obtained via Clevenger distillation from red algae species *Laurencia obtusa* and *Ceramium* spp., collected from the coastal areas of Sinop (Black Sea), were analyzed by GC/MS. Additionally, hexane, dichloromethane, chloroform, and methanol extracts were prepared using the Soxhlet extraction method, and their antileishmanial activities were evaluated. As a result, 80.92% and 90.51% of the volatile oil compositions of *Laurencia obtusa* and *Ceramium* spp. were identified, respectively, with 20 compounds characterized in each species. Hydrocarbons were the predominant compound class in the essential oils of *Laurencia obtusa* (79.31%) and *Ceramium* spp. (72.89%). The leishmanicidal activities of the solvent extracts were tested in vitro against *Leishmania infantum* promastigotes. The minimum inhibitory concentration (MIC) values for *Laurencia obtusa* extracts in hexane, dichloromethane, chloroform, and methanol were 5, 2.5, 2.5, and 10 mg/mL, respectively. For *Ceramium* spp., the MIC values were 2.5, 2.5, 2.5, and 10 mg/mL, respectively. Dichloromethane and chloroform extracts of both species exhibited greater antileishmanial activity compared to methanol and hexane extracts, indicating their higher potential as antileishmanial agents.

INTRODUCTION

Algae are primitive organisms that can be either prokaryotic or eukaryotic and contain photosynthetic pigments. They include unicellular microalgae and complex multicellular macroalgae. Physiologically, algae differ from higher plants due to the absence of specialized organs. Globally, approximately 9,000 species of seaweed—classified as either microalgae or macroalgae—are grouped based on pigmentation into brown (Phaeophyta), red (Rhodophyta), and green (Chlorophyta) algae. In Türkiye, around 5,000 algal species have been recorded, including approximately 600 with documented bibliographic and distribution data.

Red algae are predominantly marine, with only about 3% of the over 5,000 known species found in freshwater environments (Zerrifi *et al.*, 2018). Red algae (Rhodophyta) represent one of the oldest groups of eukaryotic algae and comprise one of the largest algal phyla, with over 7,000

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recognized species across more than 900 genera. These algae are primarily found in marine habitats (Dodds & Whiles, 2019), with approximately 5% occurring in freshwater, particularly in warmer climates.

Algae perform photosynthesis to produce their own food and release oxygen into the water. They also form the base of the marine food web, supporting organisms like shrimp and jellyfish. Additionally, algae contain a range of bioactive compounds such as secondary metabolites, terpenes, polyphenols, flavonoids, fatty acids, vitamins (A, B1, B2, B6, and C), and minerals (iodine, potassium, iron, magnesium, and calcium). These compounds have been utilized in traditional medicine as sedatives, muscle relaxants, and anti-edema agents. Algae are also widely used in animal feed, agricultural fertilizers, biofuel production, and polysaccharide extraction (Sanjeewa *et al.*, 2016). The red macroalga *Kappaphycus striatum*, for example, is extensively cultivated for its carrageenan content. Other red algae, such as *Palmaria palmata* (dulse) and species of *Porphyra* (laver/nori/gim), are traditional components of European and Asian diets and are used in the production of agar, carrageenan, and other food additives (Wu *et al.*, 2023).

The genus *Leishmania* (Trypanosomatida; Trypanosomatidae) comprises more than 30 species of unicellular protozoa, of which around 20 are known to be pathogenic to humans. The geographic distribution of these parasites varies, and infections can manifest in three clinical forms: Cutaneous (CL), Mucocutaneous (MCL), and Visceral (VL, Kala-Azar) Leishmaniasis (Herwaldt, 1999).

Historically, treatment of leishmaniasis has relied on pentavalent antimonials such as meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam), developed in the 1940s. Additional treatments include oral miltefosine, injectable paromomycin, and formulations of amphotericin B. However, the effectiveness of these drugs is limited, and many are associated with significant side effects, including hepatotoxicity, nephrotoxicity, and teratogenicity (Khattab *et al.*, 2017). Consequently, the search for new, safer therapeutic agents is ongoing.

Alamar Blue (resazurin) is a redox-sensitive dye commonly used to assess the viability of *Leishmania promastigotes*, allowing for the distinction between live and dead cells. Its advantages include low cost, ease of use, and long-term stability (Süleymanoğlu *et al.*, 2018).

Previous studies have investigated the antimicrobial and chemical properties of algae, including essential oil composition and biological activities. While there have been investigations into the essential oil profiles and antimicrobial activities of *Laurencia obtuse* (*L. obtusa*) in countries including Türkiye, studies on *Ceramium* spp. have largely focused on their fatty acid composition (Dellatorre *et al.*, 2020; Yesilova *et al.*, 2017). However, there is currently no data available on the essential oil composition of *Ceramium* spp. in Türkiye, nor on the antileishmanial activities of either species.

In the present study, essential oils were extracted from *L. obtusa* and *Ceramium* spp. collected from the Sinop coast of the Black Sea. Their chemical compositions were determined using GC/MS. Additionally, hexane, chloroform, methanol, and dichloromethane extracts were prepared, and their *in vitro* antileishmanial activities were evaluated.

MATERIAL and METHODS

Algae Samples

L. obtusa and *Ceramium* spp. were collected from Akliman, Sinop province, in July 2018 and placed in polyethylene bottles and brought to the laboratory under cold chain. The biomass was harvested from seawater and washed with distilled water to remove traces of sand, seaweed, and other marine organisms. The algae samples were identified by Ass. Prof. Dr. Ali KARAÇUHA, Faculty of Fisheries at Sinop University. The biomasses were dried under suitable conditions at room temperature, and size reduction was made with a steel blade blender.

Isolation of Essential Oil

The essential oil was obtained with the Clevenger mechanism. For this, the air-dried and shredded algae sample *L. obtusa* (55 g), *Ceramium* spp. (61 g) was weighed to obtain essential oil by hydrodistillation, and its storage was carried out as mentioned in the literature. The necessary procedure for the experiment was done in the literature (Yücel et al., 2023).

Extractions with Soxhlet Apparatus

The air-dried *L. obtusa* and *Ceramium* spp. were weighed at 25 g and put into the Soxhlet cartridge and placed in the refrigerator. Soxhlet extraction was performed for 24 h by adding 250 mL of hexane, dichloromethane, chloroform, and methanol to each flask. The solutions obtained at the end of the period were drawn to dryness by passing through the evaporator, and the residue was weighed and stored at +4°C until biological activity tests were performed. All solvents used are a range of 98%-95% pure and Sigma Aldrich brand (Karaman et al., 2022).

Gas Chromatography and Gas Chromatography-Mass Spectrometry Analysis

GC analysis was performed using Shimadzu QP2010 (USA) Ultra mass selective detector combined with Shimadzu 2010 Plus (USA) gas chromatography and Shimadzu 2010 Plus FID, and Shimadzu Class-5000 Chromatography Workstation software. The gas chromatography conditions were as follows: The GC oven was equipped with a RTX5M 30 m capillary column (0.25 mm ID, 0.25 µm film thickness, Restek, USA) with a carrier gas flow rate of 1.0 mL min⁻¹. The analysis started at a temperature of 60°C (2 min) and reached 240°C (5 min) at a rate of 5°C/min. The final temperature was 250°C, and the analysis was concluded by maintaining this temperature for 4 minutes. As the carrier gas (1 mL/min), the temperatures of the injector and detector were 250 °C and 230 °C, respectively. The electronic impact detection mode (EI) was applied; the ionization voltage was fixed at 70 eV, and the scan mode (40-450 m/z) was used for mass acquisition. VOCs were identified by comparing their retention indices (according to C6-C30 alkane standards) and by comparing their mass spectra with two libraries (FFNSC1.2 and W9N11). GC analysis was conducted for each sample, and the values were reported (Yücel, 2022).

Ethical Considerations

This study was an in vitro investigation conducted exclusively on a standard *L. infantum* strain (MHOM/AZ/1974/SAF-K27), obtained from a recognized culture collection. All experimental procedures were standardized and designed to ensure reproducibility. As no human subjects, animals, or fresh clinical isolates were involved at any stage, ethical approval was not required. All experiments were conducted in accordance with established biosafety protocols and best practices for in vitro microbiological research.

Parasite Culturing and Preparation

The leishmanicidal activity of extracts from *L. obtusa* and *Ceramium* spp. was evaluated against *Leishmania infantum* promastigotes using standard in vitro methods. Axenic cultures of *L. infantum* were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin (10 mg/mL), penicillin (100,000 units), and fluconazole (2 mg/mL). Cultures were incubated at 27 °C. Upon reaching the desired density, promastigotes were harvested and washed three times with phosphate-buffered saline (PBS). The cell concentration was then adjusted to 1 × 10⁶ cells/mL using a hemocytometer (Thoma slide) in RPMI-1640 medium.

In Vitro Leishmanicidal Activity Assay

Stock solutions of the extracts were prepared at concentrations identical to those used in antibacterial assays. First, 100 µL of RPMI-1640 medium containing 10% FBS was added to each well of a 96-well microplate. Then, 100 µL of each extract's stock solution was added to the first well and serially diluted (1:2) across the plate to produce a final concentration range of 0.3–10 mg/mL.

After the dilutions, 100 μ L of the *L. infantum* promastigote suspension (1×10^6 cells/mL) was added to each well. The plates were incubated at 26 °C for 20 hours. Subsequently, 20 μ L of sterile Alamar Blue® solution (0.1 mg/mL) was added to each well, and the plates were incubated for an additional 4 hours. Viability was assessed at 24, 48, and 72 hours to evaluate time-dependent effects. Amphotericin B was used as a positive control and prepared in a concentration range of 0.9–30 μ g/mL. All experiments were conducted in duplicate to ensure reproducibility and data reliability.

FINDINGS

In this study, the amounts of essential oils obtained by hydrodistillation from *Laurencia obtusa* and *Ceramium* spp. were measured as 77.2 mg and 84.5 mg, respectively. Analysis of the volatile oil compositions revealed that 80.92% of the components in *L. obtusa* and 90.51% in *Ceramium* spp. were successfully identified. A total of 22 components were detected, with the majority belonging to the hydrocarbon class. The most abundant compounds were methylcyclohexane (29.03%) in *L. obtusa* and hexane (37.06%) in *Ceramium* spp. A detailed list of the identified compounds is presented in Table 1.

Leishmania infantum promastigotes were used to determine the Minimum Inhibitory Concentration (MIC) values of *Laurencia obtusa* and *Ceramium* spp. extracts through various qualitative in vitro assays. Table 2 presents the results of promastigote growth inhibition by the algal extracts prepared in different solvents, providing a basic insight into their antileishmanial potential.

The MIC is defined as the lowest concentration of extract at which visible growth of the parasite is inhibited. This is indicated in Table 2 by a negative growth result (denoted by “–”). For *L. obtusa*, the MIC values were found to be 5 mg/mL for hexane (H), 2.5 mg/mL for dichloromethane (DCh), 2.5 mg/mL for chloroform (Ch), and 10 mg/mL for methanol (M). For instance, the hexane extract inhibited parasite growth only at 5 mg/mL but not at 2.5 mg/mL, thereby establishing the MIC at 5 mg/mL. The same interpretive principle applies across all solvents and both algal species. For *Ceramium* spp., the MIC values were as follows: 2.5 mg/mL for hexane, dichloromethane, and chloroform, and 10 mg/mL for methanol. These results demonstrate that both algal species exhibit higher antileishmanial activity in non-polar and moderately polar solvents, particularly dichloromethane and chloroform. The positive control wells, which contained no extract, exhibited parasite growth, indicated by a reddish coloration following the addition of alamarBlue®. In contrast, the negative control wells, to which no parasites were added, showed no color change in alamarBlue®, confirming the absence of contamination and validating assay integrity. Amphotericin B, used as the reference drug, demonstrated complete inhibitory activity at all tested concentrations, with an MIC of 0.9 mg/mL.

It is important to note that this qualitative method, based on the visual microscopic evaluation of parasite growth inhibition, does not necessitate statistical analysis. MIC determination is inherently a discrete, threshold-based method, where values are observed as definitive growth or no growth outcomes. As such, statistical tools like standard deviation or p-values, which are typically used to evaluate variability or compare means across groups, are not applicable in this context. Moreover, the experiments were repeated twice with consistent results, providing further support for the reliability and reproducibility of the MIC values reported.

In the study, MIC values of hexane, dichloromethane, chloroform, and methanol extracts of *L. obtusa* were found to be 5, 2.5, 2.5, and 10 mg/mL respectively. Since no extract was added to the positive control plate, the parasites continued to reproduce and the plate was detected as red. Since no parasite was added to the negative control, no change was observed in the color of alamar blue. Amphoterin B was used as the control drug and was found to be effective against the parasite at all doses. The MIC value was also found to be 0.9 mg/mL.

Table 1. Analysis of essential oils from *L. obtusa* and *Ceramium* spp.

Retention Time	Compounds	Area %(A)	Area (%) B	Exp. RI	Lit RI
Hydrocarbons					
5.124	2-methyl pentane	22.950	10.207	566	563
5.229	3-methyl pentane	15.355	8.120	575	579
5.674	Hexane	10.242	37.067	602	600
6.083	Methyl cyclohexan	29.032	15.206	720	722
6.402	1-Octene	0.562	0.465	793	792
15.260	Furan-2-pentyl	0.196	0.373	991	999
19.762	Nonane	0.024	-	1100	1100
35.805	Pentadecene	0.546	0.081	1495	1493
36.002	Pentadecane	0.287	0.483	1501	1500
43.005	Heptadecane	0.115	0.893	1701	1700
Aldehydes					
6.006	Pentanal	10.054	14.592	705	706
8.484	Hexanal	0.563	1.079	801	802
11.750	Heptanal	0.173	0.372	902	906
15.742	Octanal	0.006	0.214	1004	999
20.024	Nonanal	0.052	0.149	1105	1101
43.758	Pentadecanal	0.261	0.221	1712	1710
Alcohol					
6.335	Pentanol	0.080	-	765	771
Ketones					
11.360	2-Heptanone	-	0.303	890	892
15.248	3-Octanone	-	0.257	983	984
23.806	2-Decanone	0.050	0.192	1194	1192
Carboxylic acids					
39.804	Pentanoic acide	0.026	0.013	1603	1605
Oxygenetad sesquiterpenes					
47.805	Hexahydrofarnesyl acetone	0.405	0.261	1847	1848
Total		80.92%	90.51%		

A: *L. obtusa* B: *Ceramium* spp.**Table 2.** Growth potential of *L. obtusa* and *Ceramium* spp.

<i>Laurencia obtusa</i>	H	Ch	M	Dch
10	-	-	-	-
5	-	-	+	-
2.5	+	-	+	-
1.3	+	+	+	+
0.6	+	+	+	+
0.3	+	+	+	+
<i>Ceramium</i> spp.	H	Ch	M	Dch
10	-	-	-	-
5	-	-	+	-
2.5	-	-	+	-
1.3	+	+	+	+
0.6	+	+	+	+

H: Hexane, Ch: Chloroform, M: Methanol, Dch: Dichloromethane.

The evaluation of the growth potential of *Ceramium* spp in solvents is given in Table 2. In the study, MIC values of *Ceramium* spp. hexane, dichloromethane, chloroform and methanol extracts were found to be 2.5, 2.5, 2.5, and 10 mg/mL, respectively.

DISCUSSION and CONCLUSION

Red algae are among the oldest groups of eukaryotic algae and contain lipid-lowering polysaccharide components such as agarose, agarpectin, porphyran, and carrageenan. Agar, the first red algal product, was originally produced in the Far East and later adopted in Europe. Agarpectin, a soluble dietary fiber, has a high water absorption capacity, allowing it to swell in the stomach. This increases the feeling of fullness, delays gastric emptying, and ultimately reduces food intake, contributing to weight loss. Additionally, it can inhibit fat absorption in the body. Carrageenan, derived from the *Chondrus crispus* species, is a gel-like substance commonly used in the food industry for its thickening, binding, and emulsifying properties.

Active compounds obtained from red algae are generally halogenated and fat-soluble. Notably, the depsipeptides kahalalide A and F, extracted from *Bryopsis* species, have demonstrated in vitro activity against *Mycobacterium tuberculosis*. Research involving red algae is extensive in the literature. For instance, Karabay-Yavaşoğlu et al. (2007) reported that methanol and chloroform extracts of the red alga *Jania rubens*, widely used in the cosmetics industry, exhibited significant antimicrobial activity, whereas the volatile oils showed no notable effect (Karabay-Yavasoglu et al., 2007).

In this study, analysis of the volatile oils of the red algae species *Laurencia obtusa* and *Ceramium* spp. revealed a particularly high content of hydrocarbon compounds—79.31% and 72.89%, respectively. Aldehyde compounds were the second most abundant group, accounting for 11.11% and 16.63%, respectively. The high proportion of hydrocarbons in algal volatile oils, particularly in microalgae, can be attributed to a range of biological, ecological, and evolutionary factors. These include energy and carbon storage mechanisms, adaptation to environmental stress, genetic and enzymatic capabilities, and functional advantages in aquatic environments. Certain algal species possess highly active hydrocarbon-synthesizing enzymes, such as fatty acid decarboxylases and terpene synthases, which contribute to the elevated hydrocarbon content. These compounds act as efficient energy storage molecules for algae. In addition to the main compounds, both species contained minor quantities of pentadecanoic acid (0.026% in *L. obtusa* and 0.013% in *Ceramium* spp.) and hexahydrofarnesyl acetone (0.40% and 0.26%, respectively), representing a carboxylic acid and a sesquiterpene compound. These amounts were relatively low compared to the major components. In the antiparasitic activity assays, Amphotericin B was used as the standard drug and exhibited effectiveness at all tested concentrations, with a MIC value of 0.9 mg/mL. The MIC values of *L. obtusa* extracts in hexane, chloroform, methanol, and dichloromethane were found to be 5, 2.5, 10, and 2.5 mg/mL, respectively.

Demirel et al. (2011) conducted antimicrobial and antioxidant activity studies on freeze-dried *L. obtusa* collected from the Aegean Sea, performing GC/MS analysis and Soxhlet extraction. Their results revealed a high proportion of terpenes and terpenoids. Although their study parallels the present work, the volatile oil compositions differ considerably. This discrepancy may be due to regional, seasonal, or environmental variations such as water temperature and composition (Demirel et al., 2011). It is thought that the reason for this is that regional differences, seasonal changes, temperature differences, and parameter changes of water are effective. Esselin et al. (2017) studied outdoor-collected *L. obtusa* samples from France and analyzed ethyl acetate extracts via GC/MS. They identified bromo-substituted terpenoids and C15-acetogenin compounds. The structures of the compounds elucidated in their study differ from those identified in the present research. In this study, it was determined that the compounds whose structures were elucidated as a result of extraction and purification

processes, as well as the terpenoid compounds obtained from GC/MS analysis, were different from the compounds found in our study (Esselin *et al.*, 2017).

Aydoğmuş *et al.* (2004) examined *L. obtusa* collected from Antalya using a chloroform–methanol (2:1) solvent system and identified a variety of halogenated secondary metabolites. The composition reported in their structural determination study is also markedly different from ours (Aydoğmuş *et al.*, 2004). König and Wright (1997) reported on the sesquiterpene content and antibacterial activity of dichloromethane extracts of *L. obtusa*, with a focus on halogenated camphor derivatives. Other studies have also emphasized brominated compounds in *L. obtusa*. These findings indicate that the compounds identified in our work are distinct from those in previous reports (König and Wright, 1997). Cortés *et al.* (2014) identified carboxylic acid compounds in ethanol and dichloromethane extracts of *Ceramium rubrum* collected in Chile through GC/MS analysis (Cortés *et al.*, 2014). Similarly, Dubber & Harder (2008) studied the antibacterial activity of methanol and hexane extracts of *C. rubrum* from the Wadden Sea (Dubber & Harder, 2008). Malmvärn *et al.* (2005) identified hydroxylated and methoxylated bromodiphenyl ether compounds in GC/MS analysis of *Ceramium tenuicorne* extracts (Malmvärn *et al.*, 2005). In terms of antileishmanial activity, many studies have focused on plant extracts. Ozgun *et al.* (2024) assessed the antileishmanial activities of ethanol, diethyl ether, ethyl acetate, methanol, and n-hexane extracts of *Heracleum persicum*, comparing them to Amphotericin B. The MIC value of the standard was 0.500 µg/mL, while extract values were 151.6, 1602.0, 229.0, 343.5, and 4708.0 µg/mL, respectively. Hexane and diethyl ether extracts demonstrated strong activity, consistent with our findings on the hexane extracts (Ozgun *et al.*, 2024).

Yıldırım *et al.* (2024) evaluated the antileishmanial activity of *Cannabis sativa* and *Hypericum perforatum* extracts in various solvents (n-hexane, chloroform, ethanol, and 60% aqueous ethanol) against *Leishmania tropica*. They reported MIC values for *C. sativa* as 1680 ± 78 µg/mL (hexane), 1880 ± 82 µg/mL (chloroform), 280 ± 18 µg/mL (ethanol), and 300 ± 19 µg/mL (aqueous ethanol); and for *H. perforatum* as 4280 ± 202 µg/mL (hexane), 40 ± 3 µg/mL (chloroform), 730 ± 36 µg/mL (ethanol), and 2110 ± 152 µg/mL (aqueous ethanol). Amphotericin B had an MIC of 0.05 ± 0.002 µg/mL. These results show that the chloroform and ethanol extracts had the best activity, in agreement with our findings regarding chloroform extracts (Yıldırım *et al.*, 2024). Amang *et al.* (2023) studied the antileishmanial activity of hexane, ethyl acetate, and methanol extracts of *Khaya senegalensis* against *Leishmania donovani*, with MIC values of 2.68, 5.99, and 5.12 µg/mL, respectively, and 3.14 µg/mL for Amphotericin B. The high activity of the hexane extract further supports our findings (Amang *et al.*, 2023).

In our study, the MIC values of *Laurencia obtusa* and *Ceramium* spp. in hexane, dichloromethane, chloroform, and methanol were 5–2.5, 2.5–2.5, 2.5–2.5, and 10–10 mg/mL, respectively. These results suggest that both red algal species have potential as schistosomicidal or antileishmanial agents. *L. obtusa* displayed notably low MIC values in chloroform and dichloromethane, indicating enhanced antileishmanial activity in these solvents. Similarly, *Ceramium* spp. showed significant activity with low MIC values in hexane, chloroform, and dichloromethane. These findings suggest that both algal species are particularly effective in these solvents, whereas their biological activity is more limited in methanol.

In this study, the analysis of essential oils from *Laurencia obtusa* and *Ceramium* spp. revealed that a significant portion of the oil content consists of hydrocarbon compounds (79.27% and 72.86%, respectively). Aldehyde compounds were identified as the second most abundant group (11.09% and 16.6%). Among the terpene and terpenoid compounds, only hexahydrofarnesyl acetone was detected in small amounts (0.40% and 0.26%). These findings suggest that both algal species are promising natural sources of hydrocarbon-rich essential oils. The biological activity of organic solvent extracts from *L. obtusa* and *Ceramium* spp. was found to vary depending on the solvent used. Notably, the dichloromethane and chloroform extracts

demonstrated significant antileishmanial potential in both species. Based on these results, these red algae may be considered promising candidates for the development of natural antileishmanial agents and other pharmaceutical applications. Future studies could involve controlled experiments in laboratory animals to assess MIC values in vivo and to optimize dosage for therapeutic use.

Although current cytotoxicity data are limited, this in vitro investigation into the antileishmanial potential of *L. obtusa* and *Ceramium* spp. extracts provides a valuable preliminary insight into their pharmaceutical potential. The reproducible MIC values observed in this study serve as an important foundation for future research. We believe that these findings contribute meaningfully to the ongoing search for novel therapeutic agents against leishmaniasis and related diseases. In conclusion, while recognizing the limitations inherent in qualitative studies, we assert that our data are sufficient to justify publication and to support continued investigation. This study lays the groundwork for future research that will focus on correlation-based evaluations using isolated compounds, detailed cytotoxicity analyses, and comprehensive in vivo assessments.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Data Availability Statement

Research data is available and can be sent with e-mail upon request.

Authorship Contribution Statement

Tayyibe Beyza Yücel: Data curation, Editing manuscript, Data analysis, Literature survey, Planning, Reviewing and Editing, Validation, Writing-original draft. **Seda Fandaklı:** Data curation. **Ülkü Karaman:** Data analysis, Draft writing, Supervision, interpretation, Validation. **Şahin Direkel:** Data curation.

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