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Helminth Contamination of Commonly Consumed Raw Vegetables in Sivas Province in the Central Part of Turkey: First Molecular Detection of Human Pathogenic *Toxocara canis* Eggs in Raw Vegetables

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Abstract

Raw vegetables are the source of vitamins, minerals, proteins, and fibers that protect the human body against diseases. On the other hand, these may become sources of parasitic pathogens, which affect human health. This study aimed to assess the presence of helminth species in raw vegetables consumed in Sivas with microscopic and molecular techniques. The study material consisted of 120 vegetable samples (lettuce, parsley, peppermint, rockets, cress, and carrots) obtained from greengrocers, supermarkets, street vendors, and wholesalers. *Toxocara* spp. eggs, *T. leonina* eggs, and Rhabditiform larvae were detected in vegetable samples with a prevalence of 5.83%, 3.33%, and 24.17%, respectively. *Toxocara* spp. eggs were identified as *T. canis* using polymerase chain reaction. This is the first molecular detection of *T. canis* eggs in raw vegetables in Turkey. This study revealed that vegetables sold in Sivas are contaminated with helminth eggs or larvae. Therefore, people should take the necessary hygiene precaution, such as washing or sanitizing, before consuming these vegetables.

Keywords: Helminth, PCR, raw vegetables, Turkey, zoonosis

Introduction

Raw (fresh) vegetables are one of the most important foods in a human diet. Raw vegetables protect humans from several diseases, thanks to ingredients of vitamins, minerals, proteins, and fibers (Bekele et al., 2017; Li et al., 2020; Wagner et al., 2016). However, raw vegetables can be contaminated with parasitic pathogens due to poor hygienic procedures during production, harvesting, packing, shipping, storage, and preparation for sale (Akoachere et al., 2018; Bekele et al., 2017; Kozan et al., 2005; Li et al., 2020; Mohamed et al., 2016; Punsawad et al., 2019). Currently, the importance of food-borne parasitic pathogens such as *Toxocara* spp., *Toxoplasma gondii, Echinococcus multilocularis, Cryptosporidium* spp., and *Giardia* spp. has been increased since there is a limitation of access to safe food in the world (Bekele et al., 2017; Bouwknegt et al., 2018).

Parasitic pathogens may cause various health problems (Taylor et al., 2016; WHO, 2008), and clinical symptoms caused by parasitic pathogens might change according to the parasitic species involved. Most cases are asymptomatic, but sometimes parasitic pathogens can cause severe symptoms such as weight loss, abdominal cramps, bloody diarrhea, stomach pains, fever, vomiting, pulmonary symptoms, or neurological disorders during the migration of the larvae (Hajipour et al., 2021; Macpherson, 2013; Overgaauw & van Knapen, 2013; WHO, 2008). According to European Food Safety Authority (EFSA) report, each year at least 1000 people are exposed to different parasitic pathogens present in food in the European Union countries (EFSA, 2021). One of the most important ways of transmission of food-borne parasitic pathogens to humans is through raw vegetables (Fallah et al., 2012; Kozan et al., 2005; Mohamed et al., 2016; Punsawad et al., 2019). Several outbreaks caused by parasites have been documented due to consuming raw vegetables in recent years (Decraene et al., 2012; McKerr et al., 2015; WHO, 2008). Many studies, therefore, were performed on the determination of parasitic pathogens in raw vegetables in different parts of the world. In these studies, various helminth species were detected, such as *Ascaris lumbricoides, Toxocara* spp., *Taenia* spp., *Enterobius vermicularis*, *Hymenolepis nana,* and *Trichuris* spp. (Adamu et al., 2012; Adanir & Tasci, 2013; Amaechi et al., 2016; Avcioğlu et al., 2011; Bekele et al., 2017; El Bakri et al., 2020; Erdoğrul & Şener, 2005; Fallah et al., 2012;

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Hajipour et al., 2021; Ismail, 2016; Kozan et al., 2005; Rodrigues et al., 2020; Ulukanligil et al., 2001; Vizon et al., 2019; Yavari et al., 2019).

Microscopic diagnostic methods, like sedimentation and flotation, have been widely used by researchers in studies conducted for the determination of raw vegetable parasites (Akoachere et al., 2018; Bekele et al., 2017; Kozan et al., 2005; Rostami et al., 2016; Ulukanligil et al., 2001). But, the identification of parasites using microscopic techniques is based on only morphological features, especially egg morphology. Microscopic diagnostic methods are not eligible for identification of the species in the genus of *Toxocara* or *Taenia/Echinococcus* since these species have similar egg morphology (Adanir & Tasci, 2013; Bekele et al., 2017; Fallah et al., 2012; Kozan et al., 2005). According to researchers, species discrimination of eggs in the genus of *Toxocara* or *Taenia/Echinococcus* is done by using molecular methods, like polymerase chain reaction (PCR) (Adanir & Tasci, 2013; Li et al., 2007; Trachsel et al., 2007).

This study aimed to research the presence and distribution of helminth contamination of raw vegetables taken from greengrocers, supermarkets, street vendors, and wholesalers in the Sivas province and the first molecular identification of *Toxocara* spp. eggs obtained from raw vegetable samples in Turkey.

Methods

Study Area

This study was conducted in Sivas province, located in the Central Anatolian Region of Turkey. Sivas is the second largest province and has a geographical area of about 28,400 km^2. This city is approximately 1285 m above sea level. The city center shows continental climate features. The mean yearly temperature, precipitation, and moisture of Sivas are +8.9°C, 432 mm, and 65%, respectively (Figure 1).

Sample Collection

A total of 120 raw salad vegetables including lettuce (*Lactuca sativa*) ($n=20$), parsley (*Petroselinum crispum*) ($n=20$), peppermint (*Mentha spicata*) ($n=20$), rockets (*Eruca vesicaria*) ($n=20$), cress (*Lepidium sativum*) ($n=20$), and carrots (*Daucus carota*) ($n=20$) were obtained between December 2020 and June 2021 (Figure 2). The vegetables were collected from greengrocers, supermarkets, street vendors, and wholesalers in the Sivas province. The samples were placed into separate nylon bags to prevent cross-contamination between samples. All vegetable samples were immediately transported to the laboratory for examination in terms of helminths.

Preparation of Samples and Microscopic Examination

The vegetable samples were weighed in portions of 200 g. The carrots samples were peeled and the peeled material (about 200 g) was examined for helminth. The vegetable samples were placed into a 1.5 L detergent solution (containing 1% sodium dodecyl sulfate (SDS) and 0.1% Tween 80) for 12 hours. During this period, the material was mixed every 1 hour to make helminths eggs pass into the detergent solutions. The vegetable parts were discarded from the washing solution. The solution was left to sedimentation overnight, and then the top water was discarded carefully, without shaking the sediment. About 50 mL of the sediment was transferred to the Falcon tube and centrifuged for 15 minutes at 1500 ×*g* (Avcioğlu et al., 2011; Kozan et al., 2005). After centrifugation, the supernatant was decanted, and a part of the final sediment was examined in terms of possible trematode species eggs. Other part of the sediment was transferred to a centrifuge tube to which saturated $ZnSO_4$ (33%, density = 1.18) solution was added. The sample was centrifuged at 1500 ×*g* for 10 minutes (Taylor et al., 2016). Samples were examined in terms of helminth eggs under a light microscope using 10× and 40× objectives.

The genus/species identification of helminth eggs was done according to their morphological features like shape, thickness, and color of the shell (Taylor et al., 2016; Uga et al., 2000).

DNA Extraction From Toxocara spp. Eggs

Before genomic DNA extraction, *Toxocara* spp. eggs, present on the slide and cover glass, were collected into the microcentrifuge tube to increase genomic DNA. *Toxocara* spp. eggs were placed in a boiling water bath for 1 minute and thereafter snap-frozen in a −196°C liquid nitrogen for 1 minute. These processes were repeated at least 5 times. After this procedure, *Toxocara* spp. eggs were digested in 300 µL of lysis buffer (containing 20 mM Tris, 150 mM NaCl, 10 mM Ethylenediaminetetraacetic acid, 0.2% SDS) plus 15 µL of proteinase

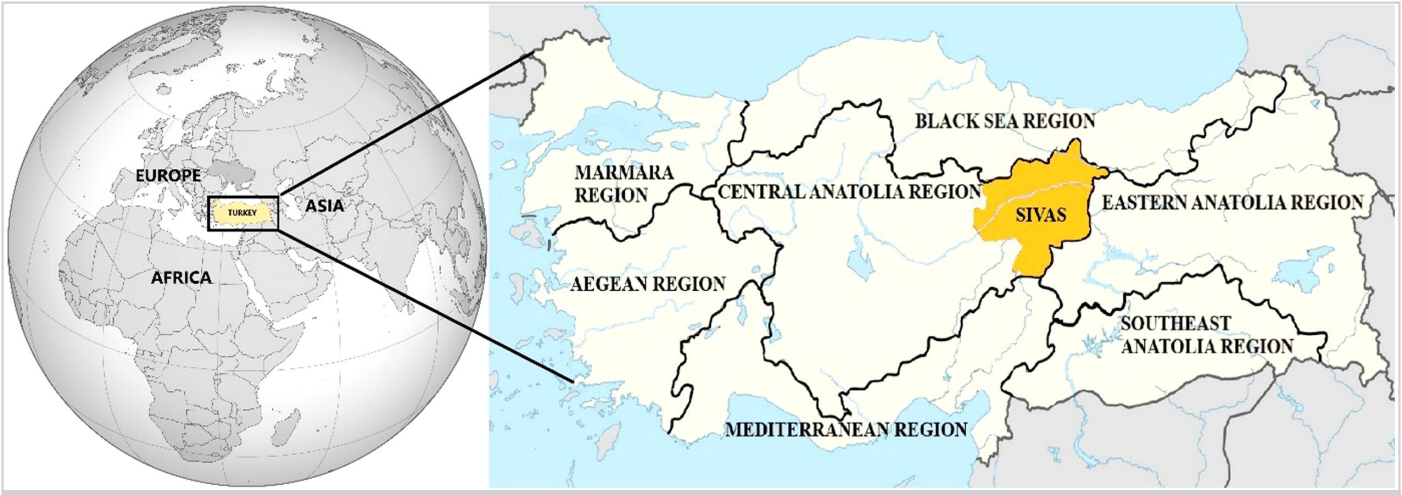

Figure 1.
Map of Turkey, Location of Sivas.

Figure 2.
Some Raw Vegetable Samples Researched in This Study. A. Peppermint, B. Cress, C. Parsley, D. Lettuce.

K (20 mg/mL (Sigma-Aldrich®, Israel)) and incubated at 56°C for 4 hours. The contents in the microcentrifuge tube were mixed and transferred to a 100°C water bath for 10 minutes for the inactivation of proteinase K. Thereafter, the final solution was mixed again, centrifuged at 13 000 rpm (16,200 ×g) for 10 minutes, and 300 µL of supernatant was used for DNA extraction using a phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v) method. The obtained genomic DNA was placed at −20°C until used in PCR procedures.

Molecular Identification of *Toxocara* spp.
Species identification of *Toxocara* spp. eggs was performed using species-specific primers that amplify the second internal transcribed spacer (ITS-2) of ribosomal DNA gene. Further data on primers are given in Table 1.

The PCR master mix was prepared as described by Erol et al. (2021) in a volume of 50 µL. The multiplex PCR was conducted as described by Erol et al. (2021). Sterile deionized water was used as negative control, and DNA of *T. canis* and *T. cati* which was obtained from adult nematodes present in our laboratory was used as positive control in each PCR assay. Approximately 15 µL of PCR products was deposited on 1.5% w/v agarose gel, and then the gel runs for 60 minutes at 90 V. After the electrophoresis process, gel was stained with ethidium bromide for 20 minutes, and specific band profiles of *T. canis* (~330 bp) and *T. cati* (~660 bp) were visualized with an ultraviolet transilluminator. All PCR protocols were done in different rooms to prevent cross-contamination.

Results

Helminths eggs were detected in 11 (9.17%) of 120 vegetables. Helminth eggs were found in lettuce (3/20), parsley (1/20), peppermint (2/20), rockets (2/20), cress (2/20), and carrots (1/20). *Toxocara* spp. eggs were detected in seven (5.83%), whereas *T. leonina* eggs were found in four (3.33%) of vegetable samples. Furthermore, rhabditiform larvae were detected in 29 (24.17%) of vegetables (Figure 3). Detailed information on helminths egg and rhabditiform larva detected in the present study is given in Table 2.

Species identification of *Toxocara* spp. eggs was done with species-specific PCR. According to PCR result, *Toxocara* spp. eggs obtained in this study were identified as *T. canis* (Figure 4).

Table 1.
Primers Used in the Present Study

Species	Primer	Length of PCR Product	Target Gene	References
T. canis	YY1 (5′-CGGTGAGCTATGCTGGTGTG-3′) NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′)	~330 bp	ITS-2	Li et al. 2007
T. cati	JW4 (5′-ACTGTCGAGGATGAGCGTGA-3′) NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′)	~660 bp	ITS-2	Li et al. 2007

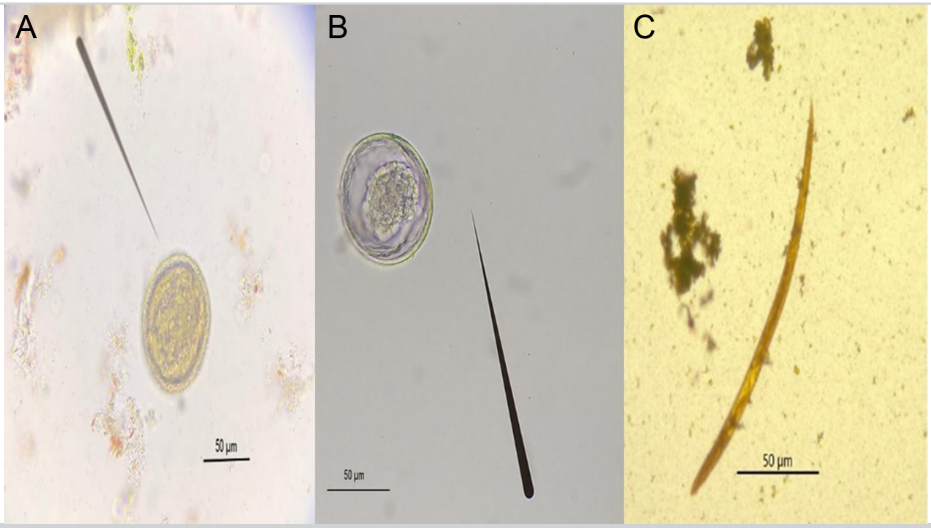

Figure 3.
The Eggs Identified in the Present Study. A. Toxocara *spp. Egg, B.* T. leonina *Egg, C. Rhabditiform Helminth Larva. A, B, and C Were Taken With an AxioCam ERc 5s (Carl Zeiss Microscopy GmbH, Germany) Camera Placed on a Primo Star Light Microscope Using ZEN 3.1 (Blue Edition) Program.*

Ethics committee certificate was not obtained in this study, since no animal experiments were performed and no animal material was used.

Discussion

Raw vegetables reduce the risk of stroke, cardiovascular diseases, and depression if regularly consumed by humans because these contain essential nutrients, such as vitamins, minerals, and fibers, necessary for human health (Liu et al., 2016; Wagner et al., 2016). On the other hand, raw vegetables may also be sources of parasitic pathogens (Bartosova et al., 2021; Hajipour et al., 2021; Healy et al., 2022; Kozan et al., 2005; Rocha et al., 2021). Different studies were done on the determination of helminth species present in raw vegetables in Turkey (Adanir & Tasci, 2013; Avcioğlu et al., 2011; Kozan et al., 2005; Ulukanligil et al., 2001). According to literature reviews, there are no data on the helminth contamination of raw vegetables in the Sivas province. The objective of the current study was to determine helminth species in raw vegetables taken from greengrocers, supermarkets, street vendors, and wholesalers in the Sivas province for the first time using microscopic and molecular techniques.

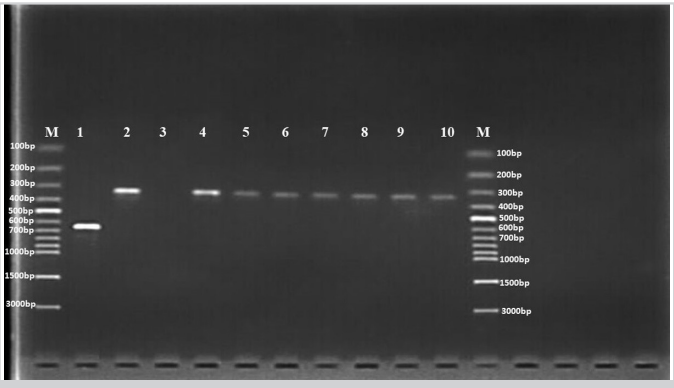

Figure 4.
Agarose-Gel Electrophoresis of Toxocara *spp.-Specific Polymerase Chain Reaction. Lane 1* = T. cati *Positive Control DNA (~660 bp); Lane 2* = T. canis *Positive Control DNA (~3300 bp); Lane 3* = *Negative Control Distilled Water; Lane 4-10* = T. canis *Positive DNA; M* = *marker.*

In this study, helminth eggs were found in 11 (9.17%) of 120 vegetables with microscopic methods. Comparison of our helminth prevalence to the values reported in different studies has a higher prevalence in Ankara (5.91%) (Kozan et al., 2005), Bursa (3%) (Avcioğlu et al., 2011), and Burdur (6.3%) (Adanir & Tasci, 2013), and a lower prevalence in Şanlıurfa (14%) (Ulukanligil et al., 2001). The positive rate in this study was lower than in some studies conducted in Thailand (35.1%) (Punsawad et al., 2019), Brazil (41.67%) (Rocha et al., 2021), and Iran (14.89%) (Rostami et al., 2016), but higher than in England (2.4%) (Healy et al., 2022) and Nigeria (3.5%) (Adamu et al., 2012). The prevalence of helminth eggs in vegetable samples may change according to irrigation methods of fields, hygienic conditions of vegetables during harvesting, transportation, and selling, the sample size of vegetables, and sensitivity-specificity of laboratory techniques used in the studies (Bekele et al., 2017; Healy et al., 2022; Kozan et al., 2005; Mohamed et al., 2016; Ulukanligil et al., 2001). Climatic conditions (average annual temperature is +8.9°C) are not fit for raising vegetables in Sivas province. Therefore, most of the vegetables come to Sivas from different provinces. These vegetables may be exposed to contaminants like helminth eggs during the storage and transportation process via humans or animals. Probably helminth eggs were found in high prevalence in vegetable samples in the current study compared to the other studies in Ankara, Bursa, and Burdur (Adanir & Tasci, 2013; Avcioğlu et al., 2011; Kozan et al., 2005).

Toxocara spp. eggs were detected in 5.83% of the vegetable samples in this work. *Toxocara* spp. eggs were found in the studies performed in Turkey, Ethiopia, Iran, Brazil, and England, ranging in prevalence between 1% and 15.83% in the vegetable samples. (Avcioğlu et al., 2011; Bekele et al., 2017; Fallah et al., 2012; Healy et al., 2022; Kozan

Table 2.
Distribution of Helminths Eggs and Larvae Detected in the Vegetable Samples

	Samples	*Toxocara* spp.	*T. leonina*	Rhabditiform Larvae
Greengrocers	Lettuce (*n* = 20)	0	0	0
	Parsley (*n* = 20)	0	0	0
	Peppermint (*n* = 20)	1	0	0
	Rockets (*n* = 20)	0	0	0
	Cress (*n* = 20)	0	2	0
	Carrots (*n* = 20)	0	0	2
Supermarkets	Lettuce (*n* = 20)	0	0	2
	Parsley (*n* = 20)	0	0	1
	Peppermint (*n* = 20)	0	1	3
	Rockets (*n* = 20)	1	1	2
	Cress (*n* = 20)	0	0	1
	Carrots (*n* = 20)	0	0	0
Street vendors	Lettuce (*n* = 20)	0	0	1
	Parsley (*n* = 20)	0	0	1
	Peppermint (*n* = 20)	0	0	3
	Rockets (*n* = 20)	0	0	1
	Cress (*n* = 20)	0	0	1
	Carrots (*n* = 20)	1	0	0
Wholesalers	Lettuce (*n* = 20)	3	0	3
	Parsley (*n* = 20)	0	0	1
	Peppermint (*n* = 20)	1	0	2
	Rockets (*n* = 20)	0	0	3
	Cress (*n* = 20)	0	0	2
	Carrots (*n* = 20)	0	0	0
Total		7	4	29

et al., 2005; Rocha et al., 2021). However, in these studies, species discrimination of *Toxocara* spp. eggs was not done by the researchers using the light microscope due to similar egg morphology (Uga et al. 2000). Therefore, species identification of *Toxocara* spp. eggs is possible by using molecular methods like PCR (Li et al., 2007). The ITS-2 gene has been successfully used in different studies for the species identification of *Toxocara* species (Choobineh et al., 2019; Erol et al., 2021; Li et al., 2007). In the current study, the ITS-2 gene-specific PCR assay was used for the identification of *Toxocara* spp. eggs obtained from different vegetable samples. According to the PCR results, all *Toxocara* spp. eggs were identified as *T. canis*. To the best of our knowledge, this is the first study in which species identification of *T. canis* eggs obtained from vegetable samples was done with PCR in Turkey (Adanir & Tasci, 2013; Avcioğlu et al., 2011; Bartosova et al., 2021; Kozan et al., 2005).

Toxocara canis is a zoonotic helminth species, and humans are the paratenic hosts of *T. canis* (Taylor et al., 2016). In this study, *T. canis* eggs were detected in lettuce (n = 3), peppermint (n = 2), rockets (n = 1), and carrot (n = 1) samples. Humans get infected with *T. canis* after ingestion of embryonated eggs present in different foods like raw vegetables (Macpherson, 2013). The infective stage of *T. canis* larvae migrates to different internal organs and can cause severe clinical infections, such as neurotoxocarosis in humans. (Macpherson, 2013; Overgaauw & van Knapen, 2013). Furthermore, *T. leonina* were detected at 3.33% (4/120) in vegetable samples in this study. These results revealed that vegetables were contaminated with domestic or wild carnivores' feces during the transportation process from the field to the seller because these animals are the definitive hosts of *T. canis* and *T. leonina*, and eggs of these parasites are present in the feces of the animals.

In the present study, rhabditiform larvae were found in 24.17% (29/120) of vegetable samples. Different developmental stages (eggs, rhabditiform or filariform larvae, and adults) of helminth species may be present in the environment, and most of them cannot cause human infection (Taylor et al., 2016). But some nematodes, such as *Strongyloides* spp., *Ancylostoma caninum*, *A. braziliense*, and *Necator americanus*, may cause severe human infections like diarrhea, constipation, abdominal pain, anorexia, and cutaneous larva migrans (CDC, 2019a,b; Taylor et al., 2016). In the life cycle of the abovementioned nematodes, rhabditiform larvae may be present as free-living larvae in the environment or in raw vegetables (CDC, 2019a,b; Punsawad et al., 2019; Taylor et al., 2016). Therefore, the rhabditiform larvae detected in the current study may belong to human pathogenic species.

Conclusion and Recommendations

In this study, the lettuce, parsley, peppermint, rockets, cress, and carrots widely consumed by humans as salad ingredients in Turkey and also in Sivas were found to be contaminated with different helminth species. *T. canis* eggs, the etiological agent of human toxocariasis, were detected in lettuce, peppermint, rockets, and carrots in this study. Moreover, *T. leonina* eggs and rhabditiform larvae were also detected in vegetables. This result revealed that vegetables can be contaminated with different helminth species during their process from field to fork. Therefore, we strongly recommend washing and sanitizing raw vegetables with sodium hypochlorite or vinegar before consumption (Amoah et al., 2007; Gomes Neto et al., 2012; Hajipour et al., 2021) to ensure protection against pathogens transmitted via this route.

Ethics Committee Approval: Ethics committee certificate was not obtained in this study, since no animal experiments were performed and no animal material was used.

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Author Contributions: Concept - U.E., K.A.; Design - U.E., K.A.; Supervision - K.A.; Materials - U.E., Ö.F.Ş., O.F.U.; Data Collection and/or Processing: U.E., Ö.F.Ş., O.F.U.; Analysis and/or Interpretation - U.E., K.A., Ö.F.Ş., O.F.U.; Literature Review - U.E., K.A.; Writing - U.E.; Critical Review - K.A.

Declaration of Interests: The authors declare that they have no competing interest.

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