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Research Article/Araştırma Makalesi

Investigation of *Toxoplasma gondii* Seroprevalence in Pregnant Women in Çankırı

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Abstract: *Toxoplasma gondii* can be transmitted to humans through consumption of raw or undercooked meat containing live tissue cysts, consumption of water or food contaminated with oocysts shed from cat feces, and vertical transmission during pregnancy or through tissue, organ, and blood transfusion. The aim of this study was to investigate the seroprevalence of *T. gondii* in patients admitted to the Obstetrics and Gynecology Department of Çankırı State Hospital (ÇSH). In this study, anti-*T. gondii* IgG and IgM antibody levels of 62 pregnant women between the ages of 17 and 47 who applied to ÇSH gynecology and obstetrics outpatient clinic for normal pregnancy follow-up between June 2022 and January 2023 were investigated. According to the results of the studies, anti-*T gondii* IgG positivity is 12.9% and negative 87.1% and anti-*T. gondii* IgM positivity is 0% while it was found to be 100% negative. According to these results, it is reported that the majority of pregnant women do not encounter *T. gondii* and; therefore, they should be more careful in terms of congenital toxoplasmosis. *T. gondii* can be transmitted to humans through consumption of water or food contaminated with oocysts excreted in cat feces and through vertical transmission or tissue, organ, and blood transfusion during pregnancy. Therefore, pregnant women should be carefully monitored for *T. gondii*.

Keywords: anti-*T. gondii* IgG, anti-*T. gondii* IgM, ELISA, Pregnant, *Toxoplasma gondii*.

Çankırı İlindeki Gebe Kadınlarda *Toxoplasma gondii* Seroprevalansının Araştırılması

Öz: *Toxoplasma gondii*, canlı doku kistleri içeren çiğ veya az pişmiş et tüketimi, kedi dışkısından atılan ookistlerin bulaştığı su veya gıdaların tüketilmesi, ayrıca hamilelik sırasında dikey geçiş veya doku, organ ve kan nakli yoluyla insanlara bulaşabilmektedir. Çalışmanın amacı Çankırı Devlet Hastanesi (ÇSH) kadın doğum servisine başvuran hastalarda *T. gondii* seroprevalansını araştırmaktır. Bu çalışmada Haziran 2022-Ocak 2023 tarihleri arasında ÇSH Kadın Hastalıkları ve Doğum polikliniğine normal gebelik takibi için başvuran 62 gebenin anti-*T. gondii* IgG ve IgM antikor değerleri araştırılmıştır. Çalışmalardan elde edilen sonuçlara göre anti-*T. gondii* IgG pozitifliği %12.9; negatif %87.1 ve anti-*T. gondii* IgM pozitif %0; negative %100 olduğu belirlenmiştir. Bu sonuçlara göre gebelerin büyük çoğunluğunun *T. gondii* ile karşılaşmadığı ve bu nedenle konjenital toxoplazmoz açısından daha dikkatli olmaları gerektiği bildirilmektedir. Gebeler tarafından *T. gondii*'nin kedi dışkısı ile atılan ookistlerle kontamine olmuş su veya gıdaların tüketilmesi ve hamilelik sırasında dikey bulaşma yoluyla veya doku, organ ve kan nakli yoluyla insanlara bulaşabildiği bilinmelidir. Bu nedenle gebeler *T. gondii* açısından dikkatle takip edilmelidir.

Anahtar kelimeler: anti -*T. gondii* IgG, anti -*T. gondii* IgM, ELISA, Gebe, *Toxoplasma gondii*.

1. Introduction

Toxoplasma gondii is a zoonotic parasitic disease seen all over the world (Liu et al., 2020; Yücesan et al., 2021). It is known that approximately one billion people are infected with this parasite today (Xiao & Yolken, 2015). Toxoplasmosis maintains its importance today since it was first described in 1908 due to its high prevalence and the serious infections it causes in pregnant and immunosuppressed people (Dubey, 1996; Shapiro et al., 2019). *T. gondii* reproduces in both sexual and asexual stages. The sexual stage occurs only in cats and felines, which are the final hosts, by the fusion of gametocytes in the intestinal epithelium. Ultimately, cats shed oocysts containing four sporozoites (Kochanowsky & Koshy 2018).

Toxoplasma gondii can be transmitted to humans through the ingestion of raw or undercooked meat containing live tissue cysts, the ingestion of water or food contaminated by oocysts excreted in cat feces, and also by vertical transmission during pregnancy or through tissue, organ, and blood transfusions. The diagnosis of toxoplasmosis is made serologically by detecting *Toxoplasma* antibodies which can also be made by mouse/cell culture inoculation experiments, histological evaluation from tissue sections, or by searching for tachyzoites in smears prepared from body fluids (Montoya, 2002).

In individuals with normal immune systems, toxoplasmosis is asymptomatic in 80-90% of cases. However, in those with conditions that cause immune deficiency, such as organ transplantation, AIDS,

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lymphoma, or leukemia, or as a result of reactivation, bradyzoites stored in the organs can become active and cause fatal diseases (Blanchard et al., 2015). Organ transplant recipients have been proven to have acquired toxoplasmosis from an infected donor. Thus, it has been evaluated that some tissues host parasites (Galvan-Ramirez et al., 2018; Renoult et al., 1997; Montoya et al., 2001; Wreghitt et al., 1989). Elevation of IgG and IFN-γ against *T. gondii* indicates an immune reaction. Therefore, when faced with immune-suppressing events such as chemotherapy, organ transplantation, or AIDS, tachyzoite may return to replication (Shariah et al., 2010; Zhao and Ewald, 2020).

Parasitemia, which develops as a result of mother's *T. gondii* infection during pregnancy, can result in abortion, still/premature birth, or congenital toxoplasmosis (Bobić et al., 2019; Galvan-Ramirez et al., 2012; Jones et al., 2003). In transplacental transmission, although the gestational age at the time of maternal infection is important, this risk can be reduced with prenatal treatment and protection (Berghold et al., 2016). Most infected newborns have no abnormalities. However, if left untreated, chorioretinitis and neurological damage may develop. Hypophrenia, epilepsy, retinochoroiditis, cardiovascular defects, and respiratory system damage may occur in children infected with *T. gondii* (Zhoun et al., 2011). Babies born with congenital toxoplasmosis infection may develop serious and potentially fatal sequelae such as intracranial calcifications, hydrocephalus, ascites, hepatosplenomegaly, pericardial or pleural effusions, hydrops fetalis, motor and hearing disorders, and chorioretinitis. Early diagnosis during pregnancy enables early initiation of treatment, thereby preventing negative clinical outcomes. Nevertheless, when determining whether to commence treatment in the neonatal period, it is imperative to assess the serological tests of infected mothers and babies (Akçalı et al., 2017; Saso et al., 2020).

2. Material and Methods

In this study, anti-*T. gondii* IgG and IgM values of 62 pregnant women aged between 17 and 47 years who applied to the Çankırı State Hospital (ÇSH) Gynecology and Obstetrics outpatient clinic for normal pregnancy follow-up between June 2022 and January 2023 were studied. Age groups of pregnant women were determined based on ten-year periods starting from the lowest age of pregnancy. The patients were divided into four age groups: 17-26, 27-36, 37-46, and over 47. The test samples were evaluated by a student from Çankırı Karatekin University (ÇAKÜ), Faculty of Health Sciences, Department of Midwifery using the blood of pregnant women taken for testing along with sociodemographic data obtained from the archive records of ÇSH. The tests were performed using some of the serum samples stored as -20 ºC blank samples in the Biochemistry archives of ÇSH. The blood samples were tested in the laboratory of Çankırı Karatekin University Center (ÇANKAM) under the supervision of supervisors and graduate students. The anti-*T. gondii* IgG and IgM values of the patients were determined using the Thermo Scientific Plate Inkubator and Thermo Scientific Multiscan Ascent Analyzer (Thermo Scientific/ USA) and anti-*T. gondii* IgG and anti-*T. gondii* IgM (Dia.Pro Diagnostic Bioprobes, Italy) ELISA kit.

This project was approved by the Çankırı Karatekin University Ethics Committee with the decision number 29 dated 23.11.2022.

Statistical Analysis: Analysis of the statistical data of this study was performed with SPSS version 23 software. Descriptive characteristics are given using frequencies and percentages.

3. Results

The sociodemographic data obtained in this study were presented in Table 1. In our study, it was determined that all pregnant women in Table 1 were married. In addition, it had been defined that pregnancies occur between the ages of 27 and 36 when pregnancy is the most common. The highest age group in which women became pregnant was found to be (62,9%). This was followed by the 17-26 (27.4%) and 37-46 (9.7%) age groups, respectively. When test positivity is evaluated according to age, the highest rate is 27-36 (9.7%) years old. It was found that this was followed by 17-26 (1.6%) years of age and 37-46 (1.6%) years of age. Anti-*T. gondii* IgM was not found positive in any patient. Most pregnant women had social security (95.2%) and the areas they lived in are urban (96.8%). In this study, it was also observed that pregnant women were mostly (77.5%) not working/housewives. The results of the tests performed on *T. gondii* were given in Table 1. At the same time, the anti-*T. gondii* IgG and anti-*T. gondii* IgM results of pregnant women were determined. The results of the studies indicated that 12.9% of the participants were positive for anti-*T. gondii* IgG, 87.1% were negative, and 0% were positive for anti-*T. gondii* IgM. It was found that 100% of the participants were negative for anti-*T. gondii* IgM.

Table 1. Sociodemographic findings and *Toxoplasma gondii* serological results in pregnant women.

Sociodemographic Information		n	%	Tests Detected Positive				Tests Detected Negative			
				Anti- *Toxoplasma gondii* IgG		Anti- *Toxoplasma gondii* IgM		Anti- *Toxoplasma gondii* IgG		Anti- *Toxoplasma gondii* IgM	
				n	%	n	%	n	%	n	%
Marital Status	Married	62	100	8	12.9	0	0	54	87.1	62	100
	Single	0	0	0	0	0	0	0	0	0	0
	Total	62	100	8	12.9	0	0	54	87.1	62	100
Age	17-26	17	27.4	1	1.6	0	0	16	25.8	62	100
	27-36	39	62.9	6	9.7	0	0	33	53.2	62	100
	37-46	6	9.7	1	1.6	0	0	5	8.1	62	100
	46 and over	0	0	0	0	0	0	0	0	0	0
	Total	62	100	8	12.9	0	0	54	87.1	62	100

Sociodemographic Information		n	%	Tests Detected Positive				Tests Detected Negative			
				Anti- *Toxoplasma gondii* IgG		Anti- *Toxoplasma gondii* IgM		Anti- *Toxoplasma gondii* IgG		Anti- *Toxoplasma gondii* IgM	
				n	%	n	%	n	%	n	%
Area of Origin	Rural	2	3.2	2	3,2	0	0	0	0	62	100
	Urban	60	96.8	6	9.7	0	0	54	87.1	62	100
	Total	62	100	8	12.9	0	0	54	87.1	62	100
Social Security	Yes	59	95.2	8	12.9	0	0	51	82.2	62	100
	No	3	4.8	0	0	0	0	3	4.9	62	100
	Total	62	100	8	12.9	0	0	54	87.1	62	100
Occupation	Not working /Housewife	48	77.5	7	11.3	0	0	41	66.2	62	100
	Employee	6	9.6	0	0	0	0	6	9.6	62	100
	Officer	8	12.9	1	1.6	0	0	7	11.3	62	100
	Retired	0	0	0	0	0	0	0	0	62	100
	Total	62	100	8	12.9	0	0	54	87.1	62	100

4. Discussion and Conclusion

Toxoplasmosis passes asymptomatic or like a flu infection in normal people. Although toxoplasmosis generally continues to be asymptomatic and the disease goes unnoticed, it actually shows that this zoonotic disease has a high seroprevalence in every region of our country (Polat et al., 2002; Kölgelier et al., 2009; Demirci & Mor 2021; Aydoğmuş et al; 2022; Görkem et al., 2022). However, it is also known that extremely severe infections occur in symptomatic cases. In cases where acute infection is suspected, a single test should not be sufficient for diagnosis (Babür et al., 2021).

This study was conducted on pregnant women who applied to ÇSH gynecology outpatient clinic. The majority of pregnant women are women who have social security (59/95.2%) and live in urban life (60/96.8%). One of the difficulties in collecting data in this study is that the entire group consists of conscious pregnant women. The rate of *T. gondii* may increase because pregnant women from rural areas are more closely associated with animals. All pregnant women were married (62/100%). The highest age group in which women became pregnant was found to be 27-36 (39/62.9%). This is followed by the 17-26 (17/27.4%) and 37-46 (6/9.7%) age groups, respectively. 27-36 years of age is the age group with the highest positivity (6/9.7%). This is followed by the 17-26 (1/1.6%) and 37-46 (1/1.6%) age groups, respectively. Anti-*T. gondii* IgG positivity was detected in all pregnant women (8/12.9%). Anti-*T. gondii* IgM positivity was not detected in 62 pregnant women. The results of this study are similar to the data of Aydoğmuş et al., (2022), Keçecioğlu et al., (2022), Durdu and Mutlu (2017), Parlak et al., (2015), and Tamer et al., (2009). Additionally, Anti-*T. gondii* IgG and Anti-*T. gondii* IgM positivity values are shown in Table 1.

The ELISA method can detect antibodies against *T. gondii*, circulating immune complex structures containing *Toxoplasma* antigens, and free *Toxoplasma* antigens. Since ELISA is an easy-to-perform and automatic system that does not require expertise, multiple samples can be tested simultaneously. In the last 20 years, granule antigens such as GRA1, GRA2, GRA4, GRA6, GRA7, and GRA8; rhoptry proteins such as ROP1 and ROP2; microneme proteins such as MIC1, MIC2, MIC3, MIC4, and MIC5; and surface antigens such as SAG1 and SAG2 have begun to be detected with ELISA methods. (Liu et al., 2015). In the ELISA method, the first marker seen in the blood of a person with acute infection is IgM. It occurs in the 1st week. It peaks in 2-3 weeks, gradually decreases, and can last up to 6-8 months. IgG type antibodies begin to appear in the 1st month. It remains at the highest titer for 6-8 weeks. It remains at the highest level for 6-8 months. It decreases to a low level in 12-18 months. Since IgA type antibodies are produced before IgMs, they can also be used as a marker in acute infections and can remain positive for months. IgE type antibodies may remain high for a very short time and may indicate acute infection (Robert-Gangneux & Dardé, 2012).

In studies, ELISA IgM, IgG, and ELISA IgG avidity methods can be used in the diagnosis of toxoplasmosis. With these methods, results can be achieved quickly and are reliable. Due to the risk of congenital toxoplasmosis, it has been proven in many studies that especially in pregnant women, in cases where ELISA gives IgM negative/positive and IgG positive results and high avidity is determined, infection occurs within 3-4 months and the method is reliable. However, it should be taken into consideration that interpreting suspicious or low avidity results using single methods may lead to incorrect results (Babür et al., 2021). In such cases, control and different verification tests are needed. In addition, knowing the avidity value along with the serological results against toxoplasmosis during pregnancy is also important in terms of excluding acute toxoplasmosis, anti-*T. gondii* IgM positivity can persist for a long time in some people. Besides, it is very important to evaluate the Toxo IgG-avidity test to determine the risk of congenital transmission in pregnant women who are being examined for toxoplasmosis and whose anti- *T. gondii* IgM and anti-*T. gondii* IgM are positive (Robert-Gangneux & Dardé, 2012).

In order to accurately evaluate the serological profiles detected in pregnant women, it is necessary to know the details about the antibody response that occurs during infection. Namely; IgA, IgE, and IgM antibodies begin to be produced in the first week after infection with *T. gondii*. While IgE disappears rapidly from the serum, IgA and IgM reach the maximum level at the end of the first month.

While IgM antibodies become negative before the sixth month in 25% of patients, they may remain positive for a year or even up to two years in other patients, depending on the sensitivity of the test method used. In some patients, IgM may become negative before three months or remain below detectable levels. IgA becomes negative more quickly than IgM; however, it is also stated that it can remain positive for up to nine months. IgG antibodies begin to become positive 1-3 weeks after IgM begins to rise, reach the maximum level in 2-3 months, and remain positive for life with titers that may vary from person to person. In line with this information, it is recommended that the IgG avidity test be performed in pregnant women with positive anti-*T. gondii* IgM and IgG tests to determine whether the infection is in the early or late stages. Although it varies depending on the method used, high IgG avidity values indicate that the person had the infection 3-5 months ago, while low avidity is considered an indicator of a new infection. However, since low-avidity antibodies can remain in the serum for months, when a low-avidity value is detected, it may not always mean a newly acquired infection. In such a case, laboratory diagnosis must be confirmed by PCR from amniotic fluid and also supported by clinical and ultrasonographic findings. In Türkiye, IgG varies between %18,9 and %82,9, IgM value varies between %0,02 and %9,87.

In this study, anti-*T. gondii* IgG and anti-*T. gondii* IgM results in pregnant women were examined. Anti-*T. gondii* IgG 12.9%; negative 87.1% and anti-*T. gondii* IgM positive 0% while it was found to be 100% negative. Avidity tests are actually extremely important for pregnant women and should be followed. By determining the IgG avidity test, if there is an infection in pregnant women, it can be predicted when it was acquired. Thus, the history of the infection becomes clear. If there are false negative results that may occur as a result of ELISA tests or suspicious and risky situations that may cause false positives due to cross reactions, in addition to serological tests, it is also reliable to apply molecular tests by laboratories with suitable infrastructure (UMS, 2015).

Table 2 shows the results of studies conducted on pregnant women in Türkiye. In Türkiye, anti-*T. gondii* IgG values range between 17.5% and 82.9%, and anti- *T. gondii* IgM values range between 0.2% and 5.4%. In our study, anti-*T. gondii* IgG and anti- *T. gondii* IgM in pregnant women were studied together. Anti-*T. gondii* IgG was found to be % 12.9 positive and anti-*T. gondii* IgM was not positive in pregnant women. Since pregnant women could not be followed up in this study, no avidity test was performed or followed up on any pregnant woman. Normally, these women should be followed by their gestational age and anti-*T. gondii* IgG and IgM and avidity tests.

Table 2. Studies carried out in Türkiye according to the provinces in alphabetical order

DATE	PLACE	LOCALIZATION	METHOD	TOTAL PREGNANT	IgG	IgM	RESOURCES
Jan 2007- Dec 2008	Adıyaman	Adıyaman 82nd Year State Hospital	ELISA	455	%48	%0.65	(Kölgelier et al., 2009)
2010-2011	Afyon	Afyon University	ELISA	565/567	%22.7	%1.6	(Gülşah et al., 2013)
01 Jan 2012- 31 Dec 2014	Afyon	Afyon University	ELISA	1284	%23.4	%1.5	(Şimşek et al., 2016)
June 2000- Dec 2003	Afyon	Kocatepe University Faculty of Medicine Hospital	ELISA	244	%30.7	--	(Yılmazer et al., 2004)
2002	Afyon	Kocatepe University Faculty of Medicine Hospital		540	%28.9	%2.5	(Altındiş & Tanır, 2002)
July 2019- June 2021	Aksaray	Aksaray Training and Research Hospital	CMIA	3218	%21	%1.4	(Çiçek et al., 2023)
Apr 2018- Marc2021	Aksaray	Aksaray Training and Research Hospital	ELISA	456	%17.1	%0.60	(Bülbül & Bekmezci, 2022)
Jan 2021- Nov 2022	Ankara	Ankara Training and Research Hospital	ELISA	1000	%82.9	%0.60	(Aydoğmuş, 2022)
27 July 1998- 03 August 1998	Ankara	SSK Ankara Maternity Hospital	ELISA	362	%30.7	--	(Maral et al., 2002)
01 April 2010- 31 June 2013	Ankara	Ankara Numune Hospital	ELISA	4758	%27.1	%0.2	(Mumcuoğlu et al., 2014)
Jan 2018- Jan 2019	Ankara_Akyurt	Akyurt State Hospital	ELISA	259	%22.4	%0.4	(Kılıç et al., 2022)
01 April 2009- 31 April 2016	Antakya	Private Mozaik Maternity and Children's Hospital	ECLIA	11564	%48.7	-----	(Çetin & Çetin, 2017)
March 2004- January 2006	Antakya	Antakya Maternity Hospital And 628 From Iskenderun Maternity Hospita	ELISA	1652	%52.1	%0.54	(Ocak et al., 2007)
Aug 2008- June 2011	Antalya	Antalya Training and Research Hospital	CMIA	7520	%33.4	%2.4	(Çekin et al., 2011)
Octo 2009- Octo 2012	Artvin	Artvin State Hospital	ELISA	1133	%30.3	%1.3	(Çeltek et al., 2014)
------	Aydın	Adnan Menderes University Medicine School	ELISA/IFA	423	%30.1	%1.3	(Ertug et al., 2005)
Jan 2014 – Dec 2014	Balıkesir	Balıkesir University Faculty Of Medicine	ELISA	2947			(Usta et al., 2018)
2017-2018	Balıkesir	Balıkesir City Hospital	ELISA	6719	%24.1	%0.46	(Keçecioğlu et al., 2022)
Dec 2011- Dec 2016	Bingöl	Bingöl Gynecology and Children's Diseases Hospital	ECLIA	10178	%63	%2	(Duran et al., 2017)
June 2012- Jan 2013	Çanakkale	Çanakkale Onsekiz Mart University Faculty Of Medicine	ELISA	196	%28.8	%2.70	(Gencer et al., 2014)
2016-2021	Çorum	Hitit University Faculty Of Medicine	ECLIA	8531	%18.9	%1	(Kahraman & Savcı, 2022)

DATE	PLACE	LOCALIZATION	METHOD	TOTAL PREGNANT	IgG	IgM	RESOURCES
01 August 2018-01 March 2019	Çorum	Hitit University Çorum Erol Olçok Gynecological Diseases	ELISA	76	25	4	(Görkem et al., 2022)
April 2008-April 2009	Denizli		ELISA	1268	%37	%1.40	(Karabulut et al., 2011)
Sep 2016- June 2018	Diyarbakır	Gazi Yaşargil Training and Research Hospital	ELISA	8175	%34.9	%1.10	(Bakacak et al., 2014)
2000-2009	Edirne	Trakya University Faculty of Medicine		1646	%31.95	%0.97	(Varol et al., 2011)
Jan 2013- Dec 2016	Erzurum	Erzurum Nenehatun Maternity Hospital	ELISA	25525	%31	%0.60	(Tanrıverdi et al., 2018)
01 August 2014 -17 August 2014	Gaziantep (Thesis)	Gaziantep Şehitkamil State Hospital (Thesis)	ELISA	150	%56.6	%2	(Eşkin, 2018)
2007-2012	Hatay	Mustafa Kemal University Research Hospital	ELISA	3340	%57	%3.60	(Okyay et al., 2013)
Jan 2013- Dec 2013	Isparta	Isparta Gynecology and Children's Diseases Hospital	ELISA	3140	%28.4	%1.8	(Akpınar et al., 2017)
Jan 2008– Dec 2011	Isparta	Süleyman Demirel University	ELISA	726	%22.7	%5.40	(Ergün et al., 2013)
Feb 2006- Feb 2006	İstanbul	Bezmialem Vakif University, Faculty of Medicine	ELISA	102	%42.9	0	(Durdu & Mutlu, 2017)
2000-2005	İstanbul	Gata Haydarpaşa Training Hospital	ELISA	4226	%26.1	%0.6	(Dündar et al., 2009)
2012-2014	İstanbul	Gata Haydarpaşa Training Hospital	CMIA	1737	%48.4	%0.7	(Selek et al., 2015)
2002	İstanbul	Istanbul University, Cerrahpaşa Faculty of Medicine	ELISA	428	%43	%0.7	(Polat et al., 2002)
Nov 2011- Feb 2013	İstanbul_ Bayrampaşa	Bayrampaşa State Hospital-Istanbul.	ELISA	2900	%31.2	%0.9	(Keskin, 2013)
Sep 2013- Jan 2015	İstanbul_Haydarpaşa	Haydarpasa Training and Research Hospital	ELISA	1101	%31	0	(Numan et al., 2015)
Jan 2008- Jan 2013	İst-Bakırköy	Bakırköy Training and Research Hospital	ELISA	2011	%31.4	%0.80	(Doğan et al., 2014)
Jan 2012- Dec 2013	Kahramanmaraş	Kahramanmaraş Sütçü İmam University	ELISA	11324	%47.15	%2.26	(Bakacak et al., 2014)
Jan 2012- Jan 2021	Kahramanmaraş	Necip Fazıl City Hospital		29.424	%41	%1.60	(Hansu et al., 2021)
Sep 2018-March 2019	Kars	Kafkas University	ELISA	308	%44.8	%0.3	(Demirci & Mor, 2021)
Jan 2018- Jan 2022	Kastamonu	Kastamonu Training and research Hospital	CIAM	1294	%20.3	%1.10	(Tüfekçi et al., 2022)
01 Jan 2017–01 Jan 2018	Kayseri	Kayseri Training and research Hospital	ELISA	10.200	%28.9	%1	(Madendağ et al., 2018)
Jan 2006- Dec 2008	Kayseri	Kayseri Maternity Hospital	ELISA	1813/1676	%33.9	%2.5	(Kayman & Kayman, 2010)
--------	Kırıkkale	Kırıkkale University Faculty of Medicine Hospital	ELISA	310	%38.4	%0.97	(Aksoy et al., 2005)
March 2005-January 2007	Kocaeli	Kocaeli University Hospital	ELISA	1972	%48.3	%0.4	(Tamer et al., 2009)
01 March 2017- 28 Feb 2022	Konya	Meram Medical Faculty Hospital	ELFA	982	%28.30	%9.87	(Ezer et al., 2023)
April 2013-Nov 2013	Konya	Konya Ereğli State Hospital	CMIA	419	%30.3	%0.20	(Gündem & Ağır, 2014)
April 2004-Nov 2005	Malataya	İnönü University Faculty of Medicine Turgut Özal Medical Center	ELISA/IFA	312	3.trimesterde %37.5		(Doğan et al., 2012)
August 92-August 95	Malatya	İnönü University Faculty of Medicine	ELISA	510			(Kafkaslı et al., 1996)
2012-2017	Mersin	Mersin University Faculty of Medicine		3474		%7.66	(Durukan & Killi, 2019)
Jan 2019- Dec 2019	Mersin	Mersin Erdemli State Hospital	ELFA	1832/1844	%28.7	%0.70	(Gonca et al., 2021)
2012-2020	Muğla	Sıtkı Koçman University Training and Research Hospital		5158/5728	%21.6	%1.60	(Kıncı et al., 2023)
01 Nov2018-14 May 2020	Muş	Muş State Hospital	ELISA	6435	%28.9	%2.2	(Ceylan & Benli, 2009)
01 Jan 2012- 01 Jan 2014	North-Western Türkiye	_____	CMIA		%43.9	%2.5	(Aynioglu et al., 2015)
01 July 2013-01 July 2014	Ordu	Ordu University Faculty of Medicine Training and Research Hospital	ECLIA-CMIA	2791	%27.6	%1.60	(Çalgın et al., 2017)
31 July 2009-01 August 2014	Rize	Recep Tayyip Erdoğan University Faculty of Medicine	CMIA	325/1046	%41.1	%1.1	(Şentürk et al., 2015)
2018-2019	Sivas	Sivas Cumhuriyet University Faculty of Medicine Hospital	ELISA	1.150	%26.7	%1.3	(Çubuk et al., 2020)
01 Jan 2007- 31 Dec 2009	Şanlıurfa	Şanlıurfa Gynecology and Obstetrics Hospital	CMIA	12084	%68.9	%2.8	(Çiçek et al., 2012)
Sep 2009- Feb 2012	Tokat	Third Stage in Tokat Province	ELISA	3162	%32	%2	(Çeltek et al., 2014)

DATE	PLACE	LOCALIZATION	METHOD	TOTAL PREGNANT	IgG	IgM	RESOURCES
Jan 2014- Dec 2018	Trabzon	Trabzon Kanuni Training and Research Hospital	ELISA	15985	%25.9	%1.53	(Kulaksız et al., 2021)
----	Urfa	Obstetrics And Gynecology Department	ELISA	1149	%60.4	%3	(Harma et al., 2004)
01 July 2010-30 June 2011	Uşak	Uşak State Hospital	ELISA	1465	% 18.3	%3.0	(Toklu, 2013)
Sep 2015- Sep 2017	Van	Van Training and Research Hospital	ELISA	300	%49.3	%0.70	(Gürbüz & Baran, 2021)
Sep 2007-August 2008	Van	Van Women's and Children's Diseases Hospital	ELISA	625	%36	%0.30	(Efe et al., 2009)
2012-2013	Van	Van Training and Research Hospital	ELISA	9809	%37.6	%1.10	(Parlak et al., 2015)
Sep 2015 – Sep 2017	Van (Thesis)	Van Yüzüncü Yıl University Dursun Odabaş Medical Center (Thesis)	ELISA	300	%56.3	%7.6	(Hazan, 2018)
Jan 2012- Dec 2012	Yozgat	Sorgun State Hospital	ELFA	804	%36.9	% 0.2	(Kiriş Satılmış et al., 2014)

Conclusion

We think that with the results of this study, *T. gondii*, which is an important issue especially for pregnant women, will fill an important gap in Çankırı province. We think that this study should be repeated with more pregnant women.

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Research Article/Araştırma Makalesi

Effects of Herbal Safflower Oil on Longevity and Oxidative Stress

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Abstract: Safflower (*Carthamus tinctorius* L.) is one of the oldest cultivated plants. Safflower oil, separated from its seeds, has superior properties than many vegetable oils. In this study, it was aimed to determine the toxic, antitoxic or antioxidant effects of safflower oil. For this purpose, 72±4-hour old larvae of the *Drosophila melanogaster* (fruit fly) model organism Oregon R wild strain were used. According to the preliminary studies, application doses were determined as 0.3125%; 0.625%; 1.25%, and 2.5%. In addition, distilled water, ascorbic acid, and H_2O_2 control groups were formed and the toxic or antitoxic effects of using them separately or together on the larvae were investigated. In addition, the lifespan of individuals that matured from larvae were studied and antioxidant parameters (TAS/TOS/OSI) were examined in male individuals fed at the doses with the best results. All experimental sets were repeated three times. As a result, it was determined that Safflower oil does not cause any toxic effect on the larvae at the concentrations used; on the contrary, when used with H_2O_2, it has a reduced toxic effect. As a result of the longevity studies of safflower oil, it was observed that the longest average life was in the %1.25 Safflower Oil + H_2O_2 application group with 65±1.09 days. It was determined from the data obtained from antioxidant studies that the antioxidant capacity of safflower oil was high, but this result was not statistically significant compared to the control group Ascorbic acid ($p>0.05$).

Keywords: Antioxidant effect, *Carthamus tinctorius*, *Drosophila melanogaster*, larval mortality, life span.

Bitkisel Aspir Yağının Ömür Uzunluğu ve Oksidatif Stres Üzerine Etkileri

Öz: Aspir (*Carthamus tinctorius* L.) en eski kültür bitkilerindendir. Tohumlarından ayrıştırılan aspir yağı, birçok bitkisel yağa göre üstün özelliklere sahiptir. Bu çalışma ile aspir yağının toksik, antitoksik veya antioksidan etkilerinin belirlenmesi amaçlanmıştır. Bu amaçla *Drosophila melanogaster* (meyve sineği) model organizması Oregon R yabanıl soyunun 72±4 saatlik larvaları kullanılmıştır. Yapılan ön çalışmalarla uygulama dozları %0.3125; %0.625; %1.25 ve %2.5 olarak belirlenmiştir. Ayrıca Distile su, Askorbik asit ve H_2O_2 kontrol grupları oluşturularak ayrı ayrı ve birlikte kullanımın larvalar üzerindeki toksik ya da antitoksik etkinliği araştırılmıştır. Ayrıca larvadan erginleşen bireylerin ömür uzunlukları çalışılmış ve en iyi sonuçların alındığı dozlarda beslenen erkek bireylerde antioksidan (TAS/TOS/OSİ) parametrelere bakılmıştır. Tüm deney setleri üç kez tekrar edilmiştir. Sonuç olarak aspir yağının kullanılan konsantrasyonlarda larvalar üzerinde herhangi bir toksik etki yaratmadığı aksine H_2O_2 ile kullanıldığında toksik etkiyi azaltıcı etkinlik gösterdiği tespit edilmiştir. Aspir yağının ömür uzunluğu çalışmaları sonucunda da en uzun ortalama ömrün 65±1.09 gün ile %1.25 Aspir Yağı+H_2O_2 uygulama grubunda olduğu gözlenmiştir. Antioksidan çalışmalardan elde edilen verilerden de aspir yağının antioksidan kapasitesinin yüksek olduğu ancak istatistiksel anlamda kontrol grubu Askorbik asite göre bu sonucun anlamlı olmadığı belirlenmiştir ($p>0.05$).

Anahtar kelimeler: Antioksidan etki, *Carthamus tinctorius*, *Drosophila melanogaster*, larval mortalite, uzun yaşam.

1. Introduction

Nutrition is the ability of a human being to meet the nutrients her body needs in an adequate and balanced manner in order to live a long life (Orbay, 2019). It is also defined as the eating and drinking pattern of a person throughout her life.

Our conclusion, based on research, is that our diet has a strong impact on the development of diseases. Nowadays, the food ingredient most associated with diseases is fat. It is thought that the more fat in the diet, the more fat will accumulate in people's bodies, which will pave the way for multiple diseases along with obesity (Çakmakçı & Kahyaoğlu, 2012).

New studies have shown that healthy fats are necessary for our body and have numerous benefits for our health. The real issue is what type of fat we consume.

Animal fats are less popular for health reasons due to their high saturated fat content and varying amounts of cholesterol. People who adopt a natural and healthy diet are more likely to turn to vegetable oil sources (Yurtvermez & Gıdık, 2021). The majority of the oils produced worldwide currently consist of vegetable oils (86%) (Soylu Erşahin, 2018). List of the plants from which oil is produced from their seeds around the world, from those with the largest share in production to the least, is as follows: soybean, sunflower, cotton (cottonseed), rapeseed, peanut, sesame, safflower, castor oil, poppy, flax, hemp, jojoba, corn (corn extract), olive, palm (fruit and seed), and coconut. In our country, the plants that produce oil include sunflower, olive, safflower, poppy, sesame, rapeseed, cottonseed, soybean, peanut, and corn (Gulluoglu et al., 2017).

Safflower, with its Latin name *Carthamus tinctorius* L., belongs to the Astraceae family. It is one of the oldest plants cultivated by humans (Şeker, 2019). There are 25 species of safflower discovered in the world (Soylu Erşahin, 2018). Safflower oil is obtained from the seeds of

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the safflower plant (Genç, 2019).

Safflower oil is superior and of higher quality than many vegetable oils (Taşlıgil & Şahin, 2016) containing linoleic acid, one of the unsaturated fatty acids (Öztürk et al., 2007). Recently, it has attracted attention with its conjugated linoleic acid content, which reduces fat content and provides weight loss in a healthy way (Baydar & Erbaş, 2020).

Safflower species grown in Turkey are divided into two types as oleic and linoleic types and they have many species among themselves. BAY-ER, Linas, Olas, ASOL, Balcı, Remzibey-05, Dinçer, Yenice, Yekta, Gelendost 1 and 2. Olas, and ASOL are oleic type safflower. Linas, Balcı, Gelendost, Dinçer, Remzibey-05, Yenice, and Yekta are linoleic type safflowers. BAY-ER, on the other hand, has multiple lines and fatty acid ratios depending on the lines (Demirci, 2020).

In healthy individuals, reactive oxygen species and antioxidant enzyme activity are always kept in balance. Otherwise, various diseases and oxidative damage occur. Oxidant and antioxidant molecules can be measured with different analytical methods (Güneş, 2016a). A faster and more functional method, TAS and TOS measurement, is attracting attention (Scandalios, 2002). Erel (2005) discovered an easy and economical method for measuring TAS and TOS and calculated the oxidative index (Çobanoğlu, 2011; Güneş, 2015). The ratio of total oxidant status to total antioxidant status as a percentage gives the oxidative stress index (Kosecik et al., 2005).

Aging and long life have been a subject of people's curiosity for years. This biological process is quite complex and intricate. Free Radical Theory is the most accepted and most researched theory among aging theories (Harman, 1956). In our study, oxidative stress and longevity were investigated based on this theory.

Insect species have an important place in studies in the field of nutrition and are frequently preferred due to reasons such as the fact that insects have an important place as balance keepers in the existing ecosystem, direct interventions to nutrients can be easily realized and controlled, and also the substances to be added to foods can be easily processed (Güneş, 2016b). *Drosophila melanogaster*, colloquially known as the 'fruit fly', was first used in genetic studies in 1910 by American Thomas Hunt Morgan (1866-1945), who worked in the fields of zoology, embryology, developmental biology, and genetics (Topçu & Duran, 2021). It has continued to be used as a model organism for more than 100 years due to reasons such as being easy to raise, comfortable feeding conditions and low cost, easy reproduction to produce a large number of offspring in a short period of time, and thus increasing the reliability of the data obtained, short life cycles, and ease of observation (Hales et al., 2015; Tamtürk, 2019; Yi et al., 2021).

In this study, it was aimed to determine the toxic, antitoxic or antioxidant effects of safflower oil obtained from the safflower plant, which is not on our tables despite its frequent production in our country, regarding the importance of herbal nutrition, which is shown as the secret of a healthy and long life, and to investigate its effect on longevity.

2. Material and Methods

2.1. Material

2.1.1. Safflower oil

Safflower oil (*Carthamus tinctorius*), which is used as a food supplement in our experimental study, prepared by cold pressing without the use of any solvent and has a high value in terms of monounsaturated fatty acid (oleic acid), was obtained from a health products company named Zade Vital Pharmaceutical Inc. (Konya, Türkiye). It was stored at +4 degrees until used in the study.

2.1.2. *Drosophila melanogaster*

D. melanogaster used in our study has been propagated by hybridization at the Biological Research Laboratory of Amasya University, Faculty of Science and Letters, for years. In our experimental study, the Oregon (R) (wild type) strain of *D. melanogaster*, which has normal round, red eyes and no mutant characters, was used to determine larval mortality. *D. melanogaster* environment is at 40%-60% relative humidity, at 25±1 °C temperature and under constant dark conditions.

2.2. Method

2.2.1. Larval mortality and longevity studies

Safflower oil concentrations used in our study were determined by taking other studies using similar vegetable oil sources as an example (Ayar et al., 2021; Güneş, 2016b; Güneş & Danacıoğlu, 2018; Heinrichsen et al., 2014). Based on previous studies, the concentrations of Ascorbic Acid (ASC) and Hydrogen Peroxide (H_2O_2) control groups were determined as 20 mM and 0.02%, respectively (Bahadorani et al., 2008; Vitorovic et al., 2021). The rates of Safflower Oil (SFO) application groups were determined as 0.3125%, 0.625%, 1.25%, and 2.5% by preliminary tests.

D. melanogaster larval mortality and substance application studies were carried out in ready-made Drosophila medium (Instant *D. melanogaster* Medium, Carolina Biological Supply Company) and lifespan studies were carried out in standard Drosophila medium.

To obtain the larvae to be used in our study, ♀25 crossed adult individuals were removed from the bottles after 1 day. After mating of the adults, 3rd instar larvae, approximately 72±4 hours old, were collected (Fig. 1).

Three separate experimental groups were created to determine larval mortality. There are four separate control groups in our study. These are distilled water, ASC (ascorbic acid), H_2O_2, and ASC+ H_2O_2. Other groups consisted of our active ingredient, safflower oil-distilled water solution at different doses and safflower oil+H_2O_2 groups, which we used to compare toxic-antitoxic effects. To induce oxidative stress, 0.02% H_2O_2 was added to the nutritional medium of *D. melanogaster*. The other control group, antioxidant ascorbic acid (ASC), was used as 20 mM. The collected 3rd stage larvae were added to safflower oil-water solution dissolved in 5 ml of distilled water at the determined concentrations (0.3125%; 0.625%; 1.25%, and 2.5%), and 5 ml of control group (distilled water, ASC, H_2O_2, and H_2O_2+ASC) bottles, other groups (0.3125%SFO+ H_2O_2; 0.625%SFO+ H_2O_2; 1.25%SFO+ H_2O_2, and 2.5%SFO+ H_2O_2) were transferred to glass bottles containing *D. melanogaster* ready medium. 100 larvae were

placed in each bottle and waited to develop into adults. Adult individuals formed after approximately 1 week were transferred to small cylinder bottles and stunned with ether. The number of stunned adults was counted and recorded in a table of larval survival and mortality rates.

Figure 1. Images of individual selection and larval collection for standard cross.

Both control groups and treatment groups were kept in constant darkness, 40%-60% relative humidity, and 25±1°C oven during the experiment.

Fresh medium was prepared for the adult individuals obtained as a result of the larval mortality experiment and they were transferred to sterilized 200 ml bottles. When the media left to rest reached the appropriate consistency, these adult individuals were transferred to bottles determined according to the concentrations they were treated with. Thus, individuals were provided with nutrition throughout the lifespan experiment. The medium was renewed twice a week and the number of living adults and the number of dead adults were noted during each renewal. Dead individuals were removed from the environment. This counting continued until the last individual remained in all experimental groups and was recorded in the tables (Piper et al., 2005; Wongchum et al., 2022).

2.2.2. Biochemical analyzes (TAS/TOS)

An additional study including biochemical analyses was planned to determine the effect of safflower oil on oxidative stress markers. According to the data obtained from larval mortality and life span studies, the doses (distilled water, ASC, H_2O_2, ASC+ H_2O_2, 0.3125%, and 1.25%+ H_2O_2) that gave the best results were applied to the larvae. Adult individuals were collected approximately 1 week later. Male and female separation was done under light microscope and 10 males individuals were selected from each concentration. The collected adults were extracted by mixing with cold homogenisation buffer (1.15% KCl, 1.15% potassium chloride, 25 mM dipotassium hydrogen phosphate, 5 mM ethylene diamine tetra acetic acid, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, pH 7, 4, +4 °C) in an ultrasonic homogeniser. Sample supernatants were stored in the freezer (-18°C) until biochemical analyses were performed.

Total antioxidant status (TAS) measurement is a measurement method based on the antioxidants in the samples converting the dark blue green ABST radical into a colorless form. Commercial kits (Baran medical, Rel Assay Diagnostics) were used in the measurements and sample absorbance was measured at 660 nm (spectrophotometer Biochrom Libra S22) as specified in the kit procedure (Erel, 2004; Güneş 2016a). TAS levels (µmol Trolox Eq/L) of the samples were calculated according to the generally used standard formula (Erel, 2004). Total oxidant status (TOS) measurement is based on the color reaction of ferric ions, which are formed by the oxidants in the samples oxidizing the ferrous ion-chelator complex to ferric ions, with the chromogen substance in an acidic environment. In the measurements, a commercial kit (Baran medical, Rel Assay Diagnostics) and kit procedure were used (spectrophotometer Biochrom Libra S22). The absorbance of the samples was measured at 530 nm (Erel, 2005; Güneş 2016a). TOS levels (µmol H_2O_2 Eq/L) of the samples were calculated according to the generally used standard formula. The procedures were repeated 3 times for all samples and TOS/TAS levels and Oxidative stress index OSI were determined (Erel, 2005). The ratio of TOS to TAS was considered as the oxidative stress index (OSI). For calculation, the resulting TAS unit was converted to µmol/L and the OSI value was calculated according to the formula:

OSI (arbitrary unit) = TOS (µmol H_2O_2 equivalent/L) / TAS (µmol Trolox equivalent/L)

2.2.3. Statistical analysis of data

For the analysis of the data we obtained as a result of our study, SPSS version 27.0 (Statistical Package for the Social Sciences) program was used. For this purpose, the "One-way Analysis of Variance" (One-way ANOVA) method was applied. Duncan test was evaluated at 0.05 probability level for the data obtained from survival rates, lifespan, and biochemical studies ($p<0.05$). Larval mortality graphs of adult individuals, lifespan survival curves, and other graphs were drawn using the Microsoft Windows Office Excel program.

3. Results

3.1. Results from Larval Mortality and Lifespan Studies

First of all, from the results obtained from larval mortality studies, it was determined that the highest larval mortality rate was in the H_2O_2 control group (0.02%) and the 2.5% SFO+H_2O_2 application group (24%) (Table 1). The best survival was observed in the 1.25% SFO+ H_2O_2 application group (100%) (Fig. 2 and Table 1).

In the second stage of our study, larvae were collected with a new experimental setup and substances were applied at determined doses from larvae to adult. 100 male adults obtained from these larvae were fed on standard medium and their mortality rates were monitored throughout their lifespan. All studies were repeated 3 times and averages were taken Then, the differences between the averages obtained as a result of pairwise comparisons between the study groups and the control groups were evaluated statistically (Table 2).

When Table 2 is examined, it is seen that the maximum lifespan of distilled water, ascorbic acid (20mM), H_2O_2 (0.02%), and H_2O_2+ASC control groups was 77±1.26 and 47±0.75, respectively. It was determined as 59±1.11 and

69±1.12 days. The most interesting result here was determined in the Ascorbic acid control group. Even though the experiment was repeated many times, the result did not change and the maximum lifespan was very short in this group. Based on the appearance of the medium, it was thought that the reason for this was that ascorbic acid created a suitable environment for microorganisms and the lifespan was short due to excessive contamination; and thus, this group was not included in the statistical calculations. The highest maximum lifespan detected was 78±0.91 days in the 1.25% SFO+H_2O_2 application group and the lowest maximum lifespan was 51±1.2 days in the 2.5% SFO application group (Table 2 and Fig. 3).

Table 1. Survival and mortality rates of larvae chronically fed with different concentrations of safflower oil.

Experiment Sets	N	Mortality Rate (%)	Survival Rate (%)
Distilled Water	100	9	91
Ascorbic Acid (ASC) (20mM)	100	8	92
H_2O_2 (%0.02)	100	25	75
H_2O_2+ASC	100	6	94
%0.3125 Safflower Oil (SFO)	100	8	92
%0.625 Safflower Oil (SFO)	100	9	91
%1.25 Safflower Oil (SFO)	100	8	92
%2.5 Safflower Oil (SFO)	100	16	84
%0.3125 Safflower Oil (SFO) +H_2O_2	100	17	83
%0.625 Safflower Oil (SFO) +H_2O_2	100	7	93
%1.25 Safflower Oil (SFO) +H_2O_2	100	0	100
%2.5 Safflower Oil (SFO) +H_2O_2	100	24	76

Table 2. Lifespan data of individuals fed with different concentrations of safflower oil.

Experiment Sets	N	Max. Lifespan (days) ± S.E.	Average Lifespan (days) ± S.E.
Distilled Water	100	77±1.26^{d}	62±1.13^{d}
ASC (20mM)	100	47±0.75*	39±0.05*
H_2O_2 (%0.02)	100	59±1.11^{a}	45±1.13^{b}
H_2O_2+ASC	100	69±1.12^{c}	52±1.13^{c}
%0.3125 SFO	100	76±1.12^{d}	61±1.11^{d}
%0.625 SFO	100	57±1.25^{a}	37±0.09^{a}
%1.25 SFO	100	68±1.26^{c}	51±0.92^{c}
%2.5 SFO	100	51±1.26^{a}	35±0.46^{a}
%0.3125 SFO+H_2O_2	100	59±0.88^{a}	42±0.88^{b}
%0.625 SFO+H_2O_2	100	61±0.91^{ab}	47±0.85^{bc}
%1.25 SFO+H_2O_2	100	78±0.91^{d}	65±1.09^{d}
%2.5 SFO+H_2O_2	100	66±1.16^{d}	52±1.16^{c}

N: Total number of individuals; Max: Maximum; ASC: Ascorbic acid; SFO: Safflower Oil; SE: Standart error; $^{a\text{-}d}$The values of the experimental groups shown with different letters in the same column are significant at the $p<0.05$ level. Groups were compared among themselves. *Due to contamination, the lifespan was thought to be short and was not included in the statistical calculations.

When looking at the average lifespans, the longest average lifespan is again in the 1.25% SFO+H_2O_2 application group and the lowest average lifespan is 65±1.09 and 35±1.09%, respectively, in the 2.5% SFO application group. It was determined as 0.46 days (Table 2, Figs. 3-6).

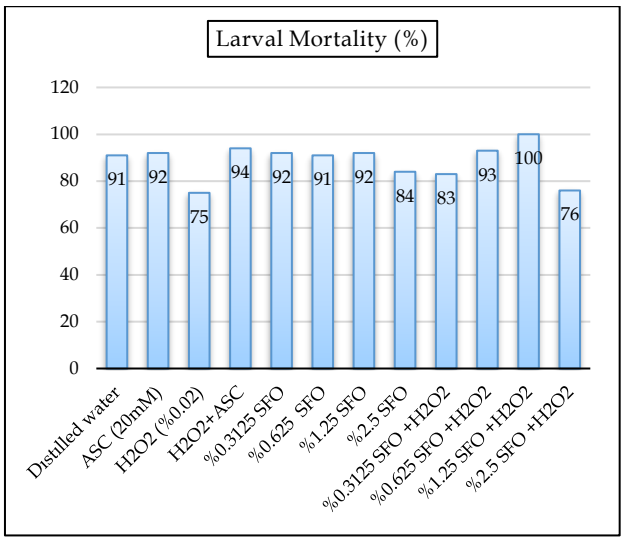

Figure 2. Survival rates of larvae fed with safflower oil.

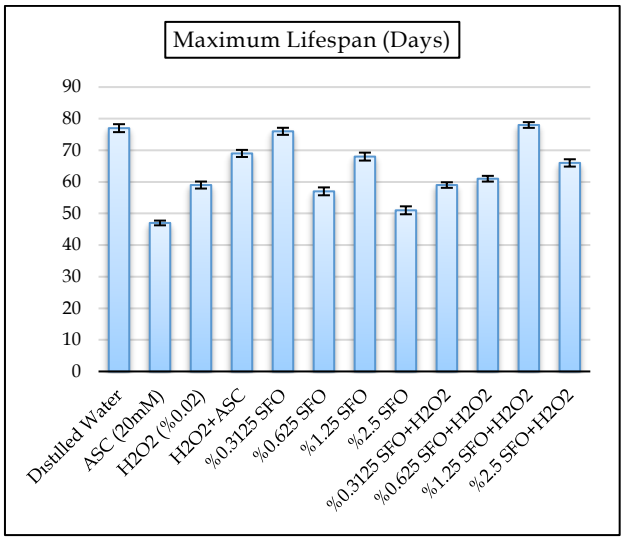

Figure 3. Maximum lifespan data of individuals fed with different concentrations of safflower oil.

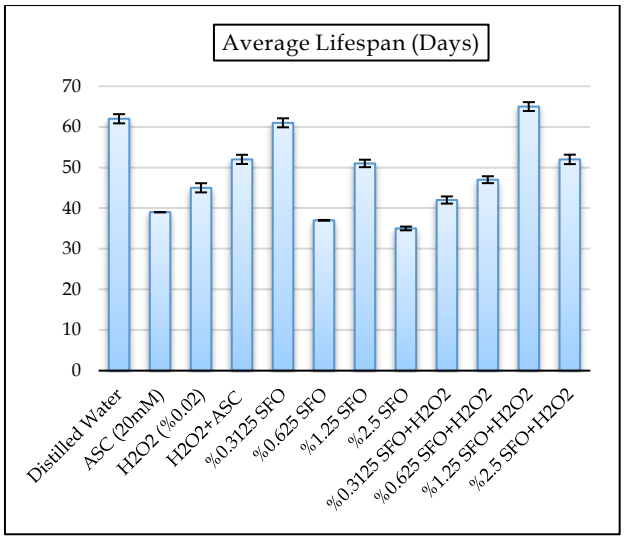

Figure 4. Average lifespan data of individuals fed with different concentrations of safflower oil.

It was determined that safflower oil has an antitoxic effect by inhibiting the toxic effect of H_2O_2 and has a life-extending effect, although not at all concentrations. In fact,

the results obtained in the 1.25% SFO+H_2O_2 application group were found to be more effective than the distilled water control group and ASC+H_2O_2 application groups. The differences obtained were also found to be statistically significant ($p<0.05$) (Table 2).

However, at higher safflower oil concentration (2.5% SFO+H_2O_2), distilled water showed a life-shortening effect, not a life-extending effect, compared to the control group.

In summary, from all the data obtained, we can say that safflower oil generally showed inhibitory activity on the toxic effect induced by H_2O_2 (Table 2, Figs. 3-6).

3.2. Results Obtained from Biochemical Analyzes

In order to determine the biochemical activity of safflower oil, the doses that gave the best results according to the larval mortality and life span data obtained as a result of our study were used (0.3125% SFO and 1.25% SFO+H_2O_2). Biochemical measurements were made on male individuals that matured from larvae fed with safflower oil and control substances at specified doses.

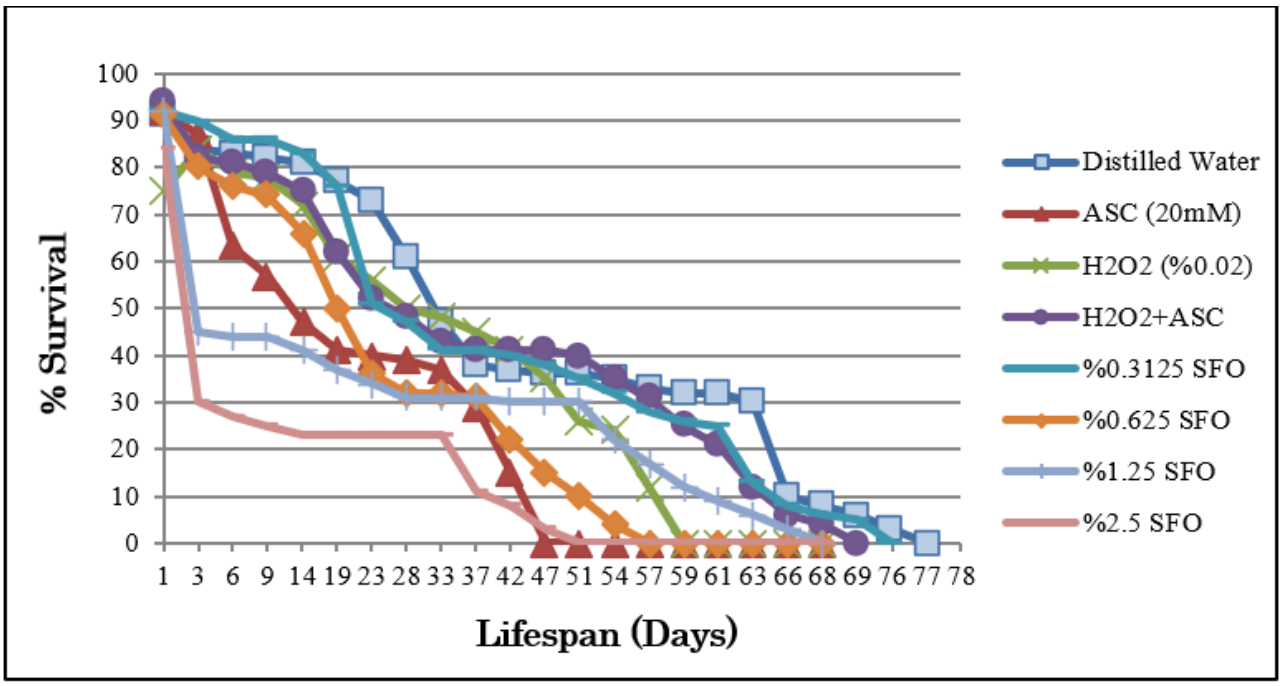

Figure 5. Lifespan curves of individuals fed and not fed with different concentrations of safflower oil.

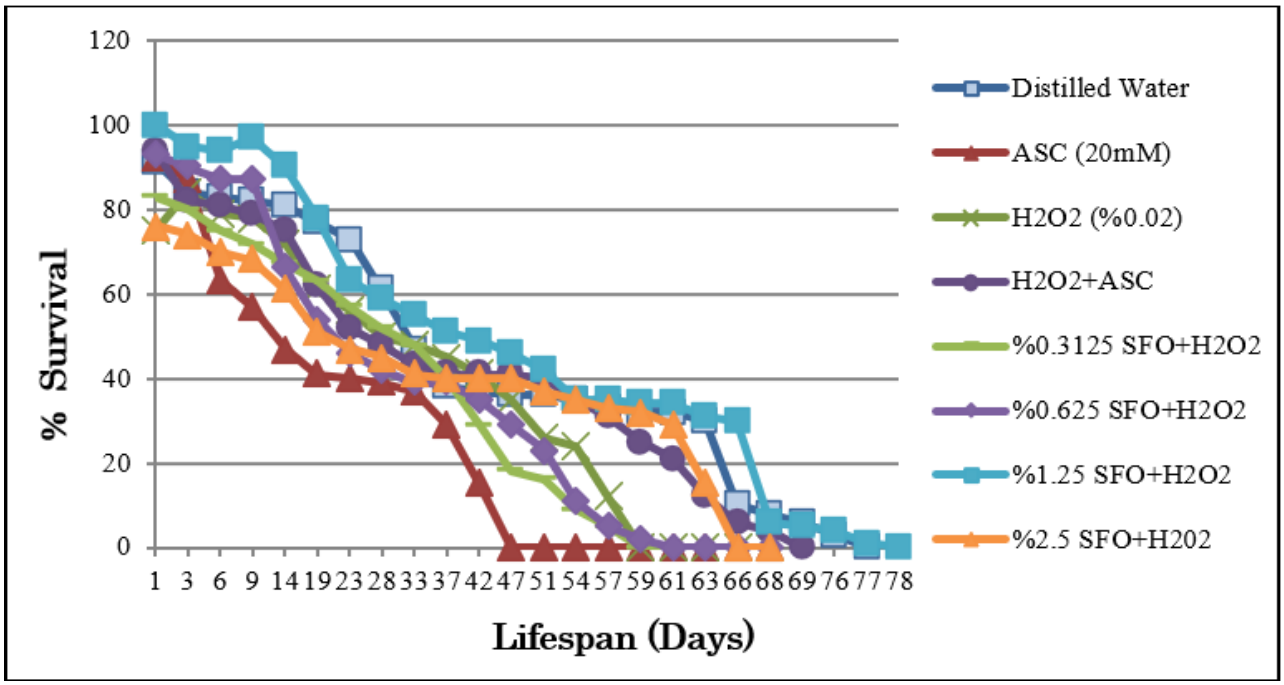

Figure 6. Lifespan curves of adult individuals chronically fed with different concentrations of safflower oil and H_2O_2.

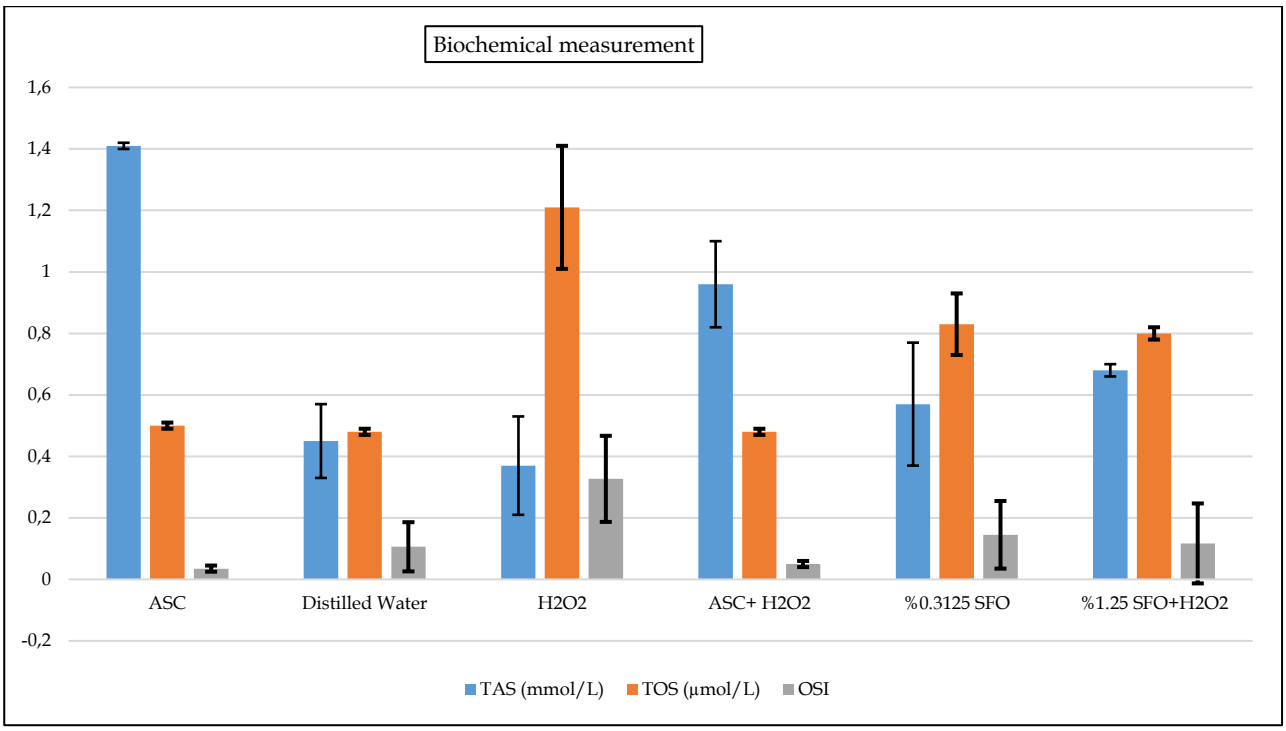

Figure 7. Biochemical data obtained from adult individuals fed with safflower oil (SFO)

When the biochemical analysis results were examined, it was observed that the highest antioxidant status (TAS) was in the ascorbic acid group (1.41 mmol/L) and the lowest was in the H_2O_2 group (0.37 mmol/L) (Table 3 and Fig. 7). However, although an increase in TAS values was observed in the safflower oil application groups compared to the negative control group, this increase remained quite low compared to Ascorbic acid and was not statistically significant ($p>0.05$).

Table 3. Comparison of biochemical data obtained from adult individuals fed with safflower oil (SFO).

Experiment Set	TAS (mmol/L)	TOS (µmol/L)	OSI
ASC	1.41±0.01^{d}	0.50±0.01^{a}	0.035±0.01^{a}
Distilled Water	0.45±0.12^{ab}	0.48±0.01^{a}	0.106±0.08^{b}
H_2O_2	0.37±0.16^{a}	1.21±0.20^{c}	0.327±0.14^{d}
ASC+ H_2O_2	0.96±0.14^{b}	0.48±0.01^{a}	0.050±0.01^{a}
%0.3125 SFO	0.57±0.20^{c}	0.83±0.10^{b}	0.145±0.11^{c}
%1.25 AY+H_2O_2	0.68±0.02^{c}	0.80±0.02^{b}	0.117±0.13^{b}

TAS: Total Antioxidant Status; TOS: Total Oxidant Status; OSI: Oxidative Stress Index; ASC: Ascorbic acid; SFO: Safflower Oil; $^{a\text{-}c}$The values of the experimental groups shown with different letters in the same column are significant at the $p<0.05$ level. Groups were compared among themselves.

When total oxidant status (TOS) was examined, the highest level was determined in the H_2O_2 group and the lowest level was determined as 1.21 µmol/L and 0.50 µmol/L in the ascorbic acid group, respectively (Table 3 and Fig. 7).

When the OSI values were examined, it was observed that the highest stress level was in the H_2O_2 application group and the lowest stress level was in the ASC and ASC+ H_2O_2 application groups (Table 3 and Fig. 7).

4. Discussion

The aim of our study is to investigate in vivo the effects of safflower oil, which we think may have high antioxidant potential, against inflammation caused by animal and saturated fat sources, which are consumed extensively in our country. It is aimed to popularise safflower oil that is very easy to grow and produce in our country but not widely used as cooking oil in our kitchen.

While there are necessary conditions for the supply of many different oil crops in our country, oilseed production cannot meet consumption (Küçük et al., 2022). The most important import products after petroleum products are oilseeds and vegetable oils. For this reason, the need for alternative oil sources is increasing. Considering the conditions of our country, safflower plant gains importance in this regard (Aşçı et al., 2022).

Turkiye currently ranks 8th in the world in safflower production and can meet approximately 7% of the world safflower production. Although safflower production increased in our country until 2014, a decline was observed in the following years. Efficiency also decreased at this rate. As of 2022, an increase of 2-3% is expected in production (Aşçı et al., 2022).

Literature review results regarding studies conducted on various vegetable oils and many different organisms, especially safflower oil, which is an alternative vegetable oil source, and the safflower plant from which it is obtained, are given below. In the literature research, it was determined that safflower plant and its oil are the subject of studies in many research areas (Amer et al., 2021; de Souza et al., 2022; Rahimi et al., 2014; Higa et al., 2010). In line with this information, no study has been found in the literature on the effect of safflower oil on longevity; therefore, our study is unique. At the same time, other vegetable oil sources have been shown to be effective in studies on longevity and antioxidant activity (Zhang et al., 2010; Zhang et al., 2017; Geçioğlu, 2016). Studies conducted with the expectation that alternative vegetable oil sources will increase the quality of life due to their positive effects

on health parameters and that the increased quality of life will also reduce disease risk factors have occupied an important place in the literature for years. In addition, the need for alternative oil sources in production supports these studies.

In their study, Zemour et al. examined extractable methanol obtained from safflower oil grown in a semi-arid climate in terms of antioxidant activity and anti-aging effect. As a result of the study, they thought that it had these effects to a significant extent; thus, it could be a valuable source of polyphenols and have a great place in the field of cosmetics (Zemour et al., 2019).

As a result of our lifespan experiments, Safflower oil gave better results than the ascorbic acid (ASC) control group. This shows how much antioxidant effect Safflower oil has compared to ASC, which is a natural antioxidant source. It is thought that the reason for its positive effect on lifespan is due to the high unsaturated fatty acid it contains and the high level of linoleic acid.

In a study on broiler chickens, the organisms were fed a combination of safflower oil and vitamin C. As a result of the study, it was observed that this diet increased the growth rate, immunity and antioxidant capacity of chickens (Amer et al., 2021).

In a study on the maternal period, safflower oil was given to mother rats. Its effect on reflex maturation, memory, and offspring hippocampal oxidative stress was examined. Safflower oil reduced lipid peroxidation as measured by MDA levels and increased antioxidant defense through SOD, CAT, GST, and GSH levels. The use of safflower oil supplement by the mother is effective on the baby's reflexes, cognitive development in the adult period, and also improves antioxidant mechanisms in the hippocampus (de Souza et al., 2022).

As observed in the studies mentioned above and in many other studies, including ours, safflower oil had an antioxidant effect on *D. melanogaster*, positively increasing lifespan.

Due to its high-quality content, many benefits of safflower oil have been observed by other studies. These benefits include antidiabetic, anticarcinogenic, antiatherogenic, and antiobesity effects (Nazir et al., 2021).

In another study, the hepatoprotective and hypolipidemic effects of safflower seed oil on diabetic rats were examined. Diabetes in rats was achieved with the help of 120 mg alloxan monohydrate per kg and safflower seed oil was given to diabetic rats as a single dose of 200 mg per kg for 28 days. As a result of blood measurements in rats at the end of the experiment, it was observed that safflower oil decreased blood sugar levels, TC, LDL, ALT, ALP, AST, and TGs levels and increased HDL cholesterol levels (Rahimi et al., 2014).

In a study conducted by Higa et al. (2010) to determine the capacity of dietary supplementation with 6% olive or 6% safflower oil, it was stated that safflower supplementation reduced malformation rates in maternal diabetes.

In a study of 40 3-week-old C57BL/6 mice, the mice were divided into 3 groups: control group (5% lard + 5% SFO), high lard group (45% lard + 5% SFO), and high safflower oil group (45% SFO + 5% lard). As a result of 10 weeks of application, it was observed that the safflower oil-supplemented diet strongly changed gene expression related to adipocytic adiposity and prevented diet-induced obesity (Zhang et al., 2010).

In addition to the positive effects of the studies, whether the safflower plant and its oil have any toxic effects has been a matter of curiosity and has been addressed by various studies.

In Zhang et al.'s (2017) study, flavonoids from the safflower plant were extracted and tested on rats for 4 weeks. The extract was used in 3 different doses (100 mg/kg, 300 mg/kg, and 500 mg/kg). As a result of the study, no significant toxicity was found (Zhang et al., 2017).

In a study conducted in Türkiye, the level of erucic acid contained in safflower seeds and their current properties were examined. The data obtained were found to be in accordance with the reference values. It was reported that the amount of erucic acid in safflower oil obtained from safflower seeds grown in Turkey would not have any negative effects on health (Geçioğlu, 2016).

Many vegetable oils have positive effects on *D. melanogaster* in many ways (antimutagenic, antitoxic, antioxidant) (Campos-Sánchez et al., 2007; Öz & Arıca, 2019). However, it has been observed that oil sources such as palm oil (Güneş et al., 2019), *Artemisia absinthium* L. essential oil (Mihajilov-Krstev et al., 2014), *Cymbocarpum erythraeum* (Apiaceae) essential oil (Aksakal et al., 2019), sunflower and soybean oil (Demir, 2011), and coconut oil (Heinrichsen & Haddad, 2012; Heinrichsen et al., 2014) have a toxic effect on *D. melanogaster*. Contrary to these studies, the safflower oil we used in our study showed a positive result by showing an antioxidant effect on *D. melanogaster*.

Many studies have shown that many food products and supplements extend the lifespan of *D. melanogaster* such as olive leaf (Güneş & Danacıoğlu, 2018), açai (Sun et al., 2010), blueberry (Peng et al., 2012), lutein (Zhang et al., 2014), turmeric (Abolaji et al., 2020), white tea (Ayar et al., 2021), perga (Fidan and Ayar, 2023), pineapple (Vicente-Crespo et al., 2021), apple (Wang et al., 2019), ursolic acid (Staats et al., 2019), and royal jelly (Kunugi and Mohammed Ali, 2019). Safflower oil is also one of the foods that extends life. In our study, survival rates were determined on our model organism fed during the larval period and the highest larval mortality rate was observed in the H_2O_2 application group and the best survival was observed at 1.25% SFO+ H_2O_2 concentration. Adult individuals were fed in standard medium and mortality rates were monitored throughout their lifespan and the longest maximum lifespan was found in the 1.25% SFO+H_2O_2 application group (78 days) and the longest average lifespan detected was 65 days in the same group.

Many nutrients used in studies such as chitosan (Güneş & Nizamlıoğlu, 2023), quinoa (Güneş, 2016a), and *Lupinus albus* L. (Güneş et al., 2020) have a positive effect on *D. melanogaster* and TAS and TOS levels. In our study, safflower oil showed a similar effect. When our biochemical analysis results were examined, the highest antioxidant level (TAS) was in the ascorbic acid group (1.41

mmol/L) and the lowest was in the H_2O_2 group (0.37 µmol/L). However, although an increase in TAS values was observed in the safflower oil application groups compared to the negative control group, this increase remained quite low compared to Ascorbic acid and was not statistically significant ($p>0.05$).

Again, many food components such as ellagic acid (Kharat et al., 2020), amaranth (Ndinawe & Kinyi, 2021), *Origanum compactum* (thyme) essential oil (Başer, 2022), and *Cyperus rotundus* (Wongchum et al., 2022) inhibited oxidative stress and inflammation induced by H_2O_2. Safflower oil, which we used in our study, also showed antitoxic effect by inhibiting the toxic effect of H_2O_2 at many concentrations.

As a result, although the basic mechanisms of aging and longevity are not yet fully understood, it is suggested that this process may be delayed. Today, studies conducted on humans on this subject are not sufficient. It is thought that the effectiveness of safflower oil, which has rich nutritional content, on *D. melanogaster* lifespan will make a significant contribution to the literature.

Ethics committee approval: Ethics committee approval is not required for this study

Conflict of interest: The authors declare that there is no conflict of interest.

Author Contributions: Conception – R.C., A.A.; Design – R.C., A.A.; Supervision – R.C., A.A.; Fund – R.C., A.A.; Materials – R.C., A.A.; Data Collection and Processing – R.C., A.A.; Analysis Interpretation – R.C., A.A.; Literature Review – R.C., A.A.; Writing – R.C., A.A.; Critical Review – R.C., A.A.

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Research Article/Araştırma Makalesi

Extraction and Physicochemical Characterization of Chitin from *Galeodes araneoides* (Pallas, 1772) (Arachnida: Solifugae)

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Abstract: Chitin is a biomaterial which has a high potential of use in many areas of technology. It is found as a structural material in the outer skeletons of arthropods, the cell walls of mushrooms, and the shells of marine invertebrates. Chitin is the most abundant biopolymer in nature after cellulose and contains nitrogen in its structure. In recent years, apart from traditional chitin sources, insects, arachnids, coral/crustacean eggs and even bat guano have been reported as alternative chitin sources. In this study, chitin was first extracted from external skeleton of a solifugids species, *Galeodes araneoides* (Pallas, 1772) and the isolated chitin was characterized by SEM, FTIR, XRD, and TGA. The obtained chitin has been found to have high thermal stability, nanofiber and nanoporous surface, and alpha form and it is suggested that it can be an alternative chitin source.

Keywords: Biomaterial, nanofibre, solifugid, arthropods, biopolymer.

Galeodes araneoides (Pallas, 1772) (Arachnida: Solifugae) Türünden Kitin Ekstraksiyonu ve Fizikokimyasal Karakterizasyonu

Öz: Teknolojinin birçok alanında yüksek bir kullanım alanına sahip olan kitin önemli bir biyomateryaldir. Mantarların hücre duvarında, eklembacaklıların ekzoiskelet iskeletlerinde ve kabuklarının temel yapısında bulunmaktadır. Kitin, selülozdan sonra doğada en fazla bulunan ve yapısında azot bulunduran biyopolimerdir. Son yıllarda geleneksel kitin kaynakları dışında böcekler, araknidler, mercan/kabuklu yumurtaları ve hatta yarasa guanosunun alternatif kitin kaynakları olduğu bildirilmiştir. Bu çalışmada bir böğü türü olarak bilinen *Galeodes araneoides* (Pallas, 1772) türünün ekzoiskeletinden ilk kez kitin elde edilmiştir. FTIR, SEM, TGA ve XRD değerlerine bakılarak bu kitinin karakterizasyonu yapılmıştır. Elde edilen kitinin alfa formda olduğu, termal kararlılığının yüksek, yüzey morfolojisinin nanofiber ve nanoporlardan oluştuğu görülmüş ve alternatif bir kitin kaynağı olabileceği önerilmiştir.

Anahtar kelimeler: Biyomateryal, nanofibril, eklembacaklılar, biyopolimer.

1. Introduction

Chitin is abundant in the basic structure of the exoskeletons of arthropods, the cell walls of fungi, and the shells of marine invertebrates. Chitin is also found in natural structures such as bat guano, insect and crustacean eggs, and etc. (Kaya et al., 2014a). Although chitin is a biopolymer generally found in the bodies of invertebrates, a recent study also recorded its presence in vertebrates (Tang et al., 2015). These studies show that more than 70% of all living things in the world have chitin in their structure.

It is estimated that chitin, which is an aminopolysaccharide and widely found in nature, is produced as much as annual cellulose for commercial purposes. Chitin and chitosan, one of its most important derivatives, are widely used in the industrial field because it is a natural resource, it is biodegradable, and does not cause environmental pollution, compatible with both plant and animal tissues and has no toxic effects, is a biologically functional compound, and its molecular structure is modifiable (Aranaz et al., 2009; Dutta et al., 2004). With the increase in studies on chitin and its derivatives and the emergence of new application areas in this field, there is a greater need for chitin and its derivatives and the orientation towards new chitin sources is increasing.

The order Solifugae, which belongs to the class Arachnida (Arachnida) of the arthropods, is one of the largest orders of arachnids in terms of the number of species. They are represented by 140 genera and 1075 species belonging to 12 families in the world and 15 genera and 44 species belonging to 6 families in our country. Compared to neighboring countries, our country is among the countries with the richest taxon diversity in terms of solifugids (Erdek, 2019; Punzo, 1998). As in other arthropods, solifugids have an exoskeleton made of chitin that covers the outer part of their bodies (Fig. 1).

The aim of this study is to isolate chitin for the first time in the world from *Galeodes araneoides* (Pallas, 1772), a solifugid species with a wide distribution in our country, and to characterize the obtained chitin using FTIR, TGA, XRD, and SEM analyses.

2. Method

2.1. Sample collection and identification

The specimens of *Galeodes araneoides* (Pallas, 1772) species used in this study were collected from Niğde province and its surroundings with the help of Dr. Melek ERDEK, who is an expert on these species in our country. Six specimens of *Galeodes araneoides* (Pallas, 1772) were subjected to the following steps in the laboratory to obtain the chitin.

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2.2. Chitin extraction process

Six *Galeodes araneoides* (Pallas, 1772) samples were washed, dried, and milled. Then the milled materials were processed with solutions (100 ml of 2 M HCl) to eliminate the inorganic content at 60-65 °C for 2 h. The solutions were then filtrated and the raw extracts were treated several times with pure water. After that, the isolated materials were placed in 50 mL of 1.0 M NaOH solutions to eliminate proteins. This stage lasted 16 h at a temperature of 130-135 °C. The solutions were re-filtered and re-washed with purified water. The extracts were treated in a mixture of methanol, chloroform, and water (2:1:4 ratio) for one hour at ambient temperature. This stage caused discoloration and lipids elimination. Lastly, the process was completed by drying the washed chitin materials in an oven at 60 °C for 24 h (Fig. 2).

Figure 1. General habitus of *Galeodes araneoides* (Pallas, 1772)

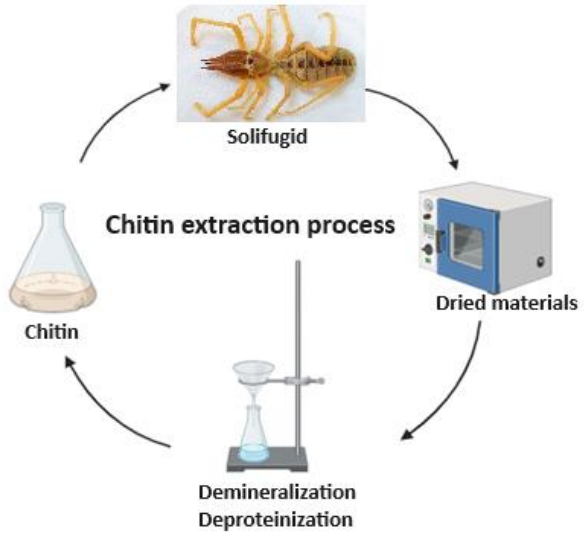

Figure 2. Scheme of chitin extraction process from *Galeodes araneoides* (Pallas, 1772)

2.3. FTIR analysis

The FTIR spectrum of the chitin sample extracted from *Galeodes araneoides* (Pallas, 1772) solifugid species were analyzed with a Bruker Vertex 70 FTIR spectrometer with a frequency range of 625–4000 cm^{-1}.

2.4. SEM analysis

The surface images of *Galeodes araneoides* (Pallas, 1772) chitin was comparatively analysed by scanning electron microscopy (Carl Zeiss, Evo LS 10). The surfaces were coated with Au by Sputter Coating System (SCS) before SEM analysis.

2.5. TGA analysis

The samples of chitin were analyzed by TGA machine (STA PT1600) with a temperature change rate of 10 °C /min from 25 to 650 °C.

2.6. XRD analysis

The samples of chitin were examined by Rigaku D max 2000 at 2θ in the range of 5-45°C. The value of crystalline index (CrI) was calculated according to formula: CrI110 = [(I110 − Iam)/I110] × 100. I110 = the maximum intensity at 2 θ = 20 °C. Iam = the intensity of amorphous diffraction at 2 θ = 16 °C (Sajomsang & Gonil 2010).

3. Results and Discussion

Chitin was obtained from *Galeodes araneoides* (Pallas, 1772) and physicochemical characterization of the obtained chitin was performed using FTIR, TGA, XRD, and SEM. In this study, chitin was obtained from solifugids of this species for the first time and was characterized.

3.1. FTIR spectroscopic analysis

In many studies, FTIR spectroscopy analysis of chitin has revealed characteristic peaks for α-chitin. These bands are: 3263 (N-H stretching), 1620 (Amide I), and 1552 cm−1 (Amide II) (Fig. 3). In this research, the chitin extracted from the *Galeodes araneoides* (Pallas, 1772) was analyzed by FTIR, two peaks at around 1650 and 1620 cm^{-1} were monitored and shown to be consistent with former studies (Fig. 3). These peaks reveal that the chitin isolated from *Galeodes araneoides* (Pallas, 1772) is in the α-form.

3.2. Thermogravimetric analysis (TGA)

Thermogravimetric analysis is widely used to learn the temperature resistance of the obtained material (Dutta et al., 2004). In the present study, TGA analysis was performed to reveal the degradation of chitin obtained from FTIR spectrum of chitin isolated from *Galeodes araneoides* (Pallas, 1772). The results obtained show that chitin experiences mass loss in two steps under the influence of temperature. In the first step, the mass losses around 100°C are due to the water that is removed from the structure. The significant mass losses observed in the second step indicate that the chitin started to degrade (Fig. 4). As a result of the literature review, mass loss of chitin isolated from different organisms was also observed in two different steps as in the present study (Jang et al., 2004; Juarez-de la Rosa et al., 2011; Sajomsang & Gonil, 2010). The maximum decomposition temperature of chitin (DTGmax) recorded for *Galeodes araneoides* (Pallas, 1772) was 334°C. This value is found lower than other arachnids DTGmax (Kaya et al., 2014a; Seyyar & Demir, 2017). These

results indicate that chitin was successfully produced from *Galeodes araneoides* (Pallas, 1772) in the present study.

3.3. X-Ray diffraction analysis (XRD)

XRD analysis results of chitin isolated from *Galeodes araneoides* (Pallas, 1772) are shown in Fig. 5. In the literature, XRD analysis of chitin and its derivatives showed two sharp peaks around 9°C and 19°C (Jang et al., 2004; Liu et al., 2012; Sajomsang & Gonil, 2010). The one around 19°C is more intense than the one around 9°C. These are the characteristic XRD peaks used in understanding of chitin and its derivatives.

XRD analysis of chitin showed two sharp peaks at 9.75°C and 19.51°C and three weak peaks at 12.93°C, 23.24°C, and 26.81°C. These peaks are very similar to the chitin peaks described in the literature (Jang et al., 2004). The crystalline index value (CrI) of chitin of *Galeodes araneoides* (Pallas, 1772) calculated as %51.14. According to the literature, the CrI values of the chitin of different arachnids were examined as 89.17% in opilionid (*Phalangium opilio*), and spiders 58.9% (*Hogna radiata*) and 78.6% (*Geolycosa vultuosa*), respectively (Kaya et al., 2014b; Seyyar & Demir, 2017). This value is lower than other arachnids. This result emphasizes that CrI value is very variable depending on species.

3.4. Scanning electron microscopy (SEM) analysis and imaging

The surface morphology of chitin of *Galeodes araneoides* (Pallas, 1772) was visualized by SEM (Fig. 6). It is seen that the surface of the chitin consists of many nanofibres and pores.

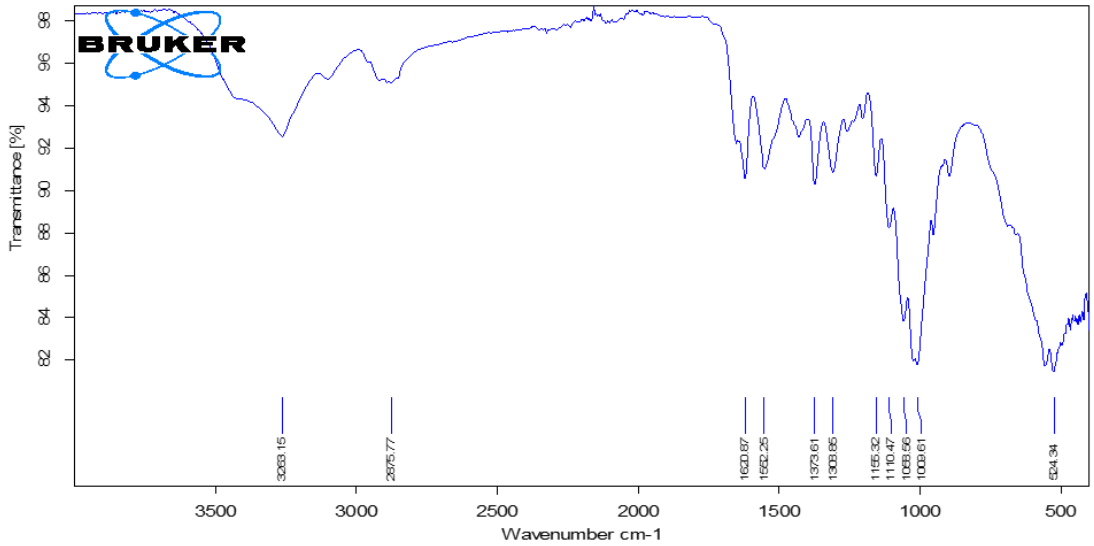

Figure 3. FTIR spectrum of chitin isolated from *Galeodes araneoides* (Pallas, 1772)

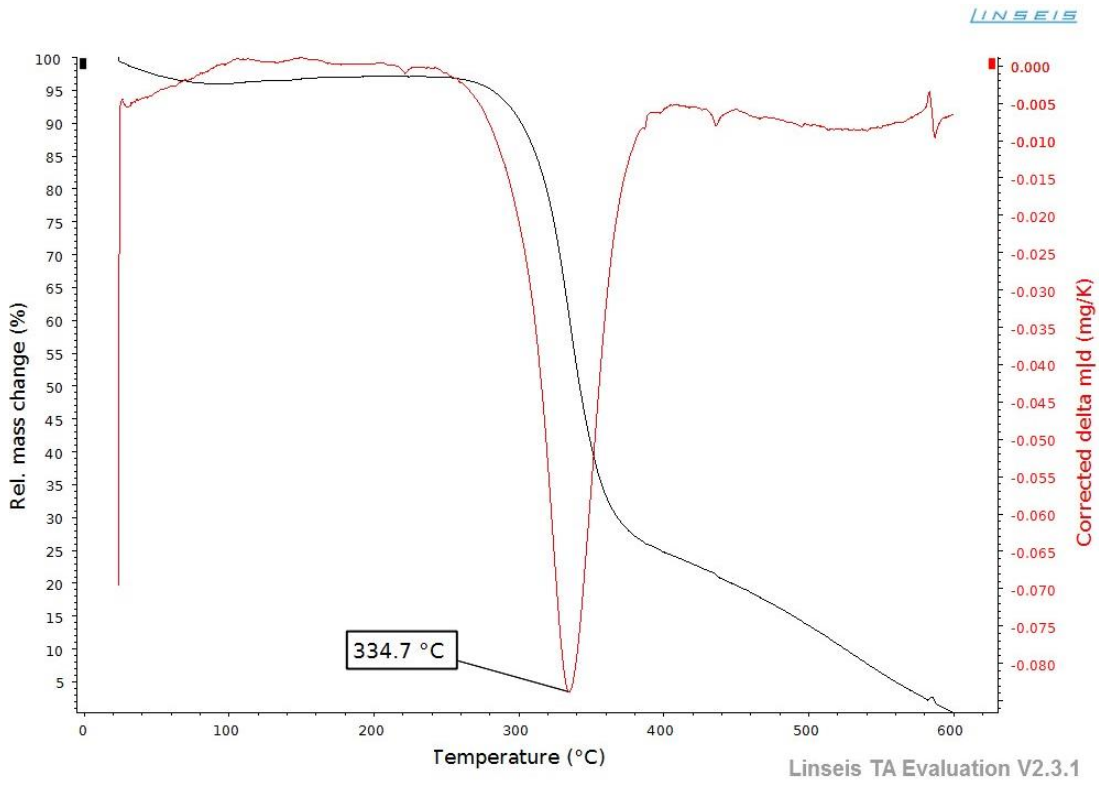

Figure 4. TGA analysis of chitin isolated from *Galeodes araneoides* (Pallas, 1772)

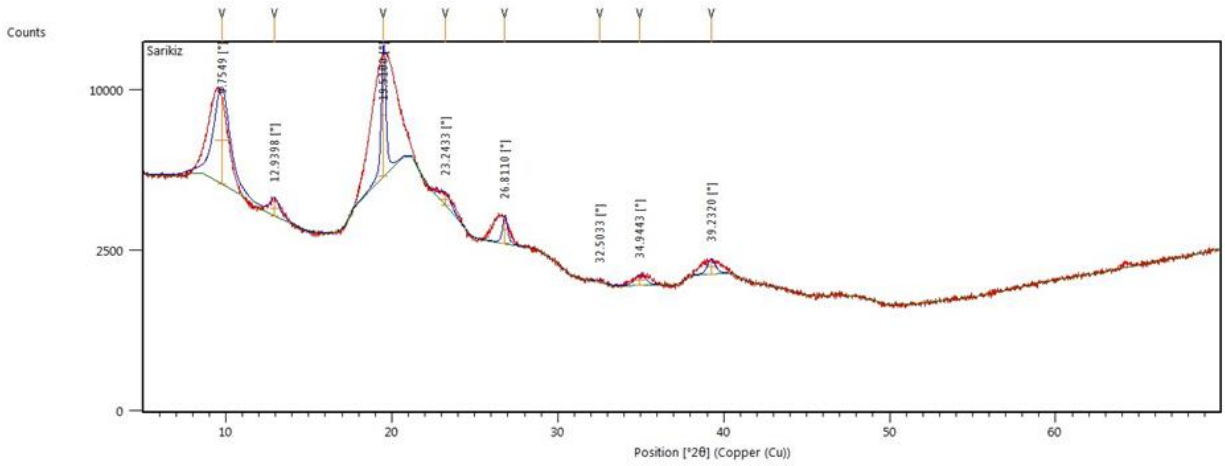

Figure 5. XRD analysis of chitin isolated from *Galeodes araneoides* (Pallas, 1772)

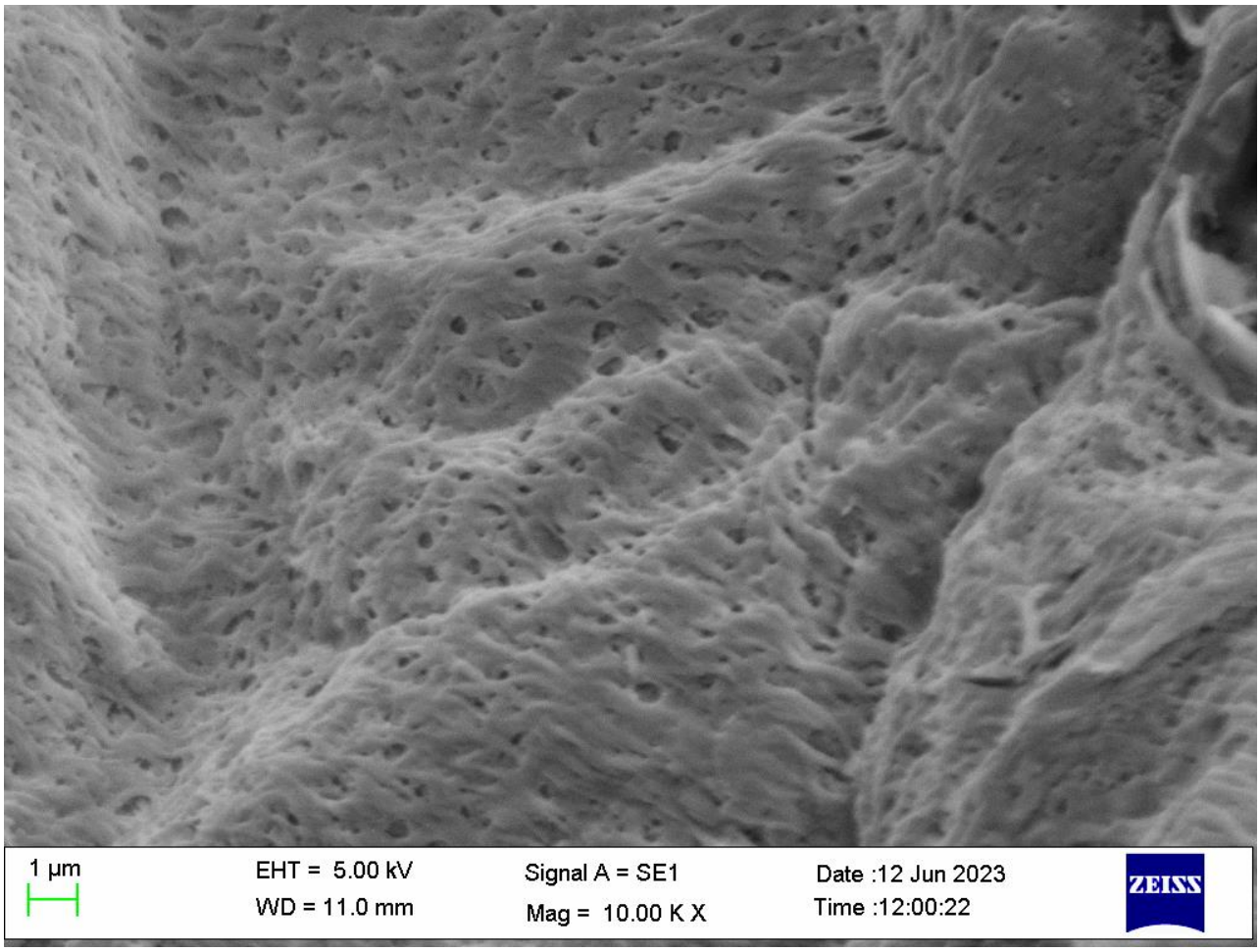

Figure 6. SEM surface morphology of chitin isolated from *Galeodes araneoides* (Pallas, 1772)

Looking at the studies in the literature, it is known that the surface of chitin varies according to the living group. During this study, it was observed that nanofibers and pores were not observed in the morphology of chitin obtained from fungi and the chitin surface was flat (Yen & Mau, 2006, 2007). It is revealed in other studies that the surface morphology of chitin obtained from some living groups is only nanofiber, while in some other living groups, the chitin surface contains both nanofibers and nanopores (Kaya et al., 2013). In very few groups of organisms, the chitin surface containing nanofibers, nanopores, and micropores together was also observed (Kaya et al., 2014b).

So far, chitin studies have only been carried out on a few species of spiders, harvestmen, and scorpion from arachnid groups in Türkiye. Among these, the chitosan surface morphology of *Mesobuthus gibbosus* (Brullé, 1832), a scorpion species, was reported to consist of dense nanofibers and pores when examined by SEM (Kaya et al., 2015, 2016). In addition, chitin was isolated from three different spider species (*Geolycosa vultuosa*, *Hogna radiata* and *Aculepeira ceropegia*) in our country and it was revealed

that they have different surface morphologies (Demir & Seyyar, 2017; Kaya et al., 2014b). In addition, in a study on opilionid species *Phalangium opilio* Linnaeus, 1758, another arachnid group, it was observed that they have a surface morphology consisting of nanofibers and nanopores (Seyyar & Demir, 2017).

4. Conclusions

The classic source of chitin isolation is the shells of crabs, shrimps, and mollusks that form waste from the processing of marine food products. In recent years, people have sought unique sources of chitin beyond these traditional sources. As a result, many fungal, insect and arachnid are now being discovered as well. In our study, we isolated chitin for the first time from solifugid which may provide a unique alternative source of chitin. Then, we investigated its characterization and some physicochemical properties obtained from *Galeodes araneoides* (Pallas, 1772) by FTIR, XRD, TGA, and SEM analyses. The findings obtained from the FTIR spectrum clearly show that the chitin obtained from this solifugid species is in alpha form. Images obtained by scanning electron microscopy show that the surface morphology of the chitin consists of nanofibers and nanopores. Thermogravimetric analysis revealed that the thermal stability of the obtained chitin was lower than that of many known insects and crustaceans. This isolated chitin has wide applications in many different industries. Therefore, we propose that solifugids can also be used as an alternative source to extract chitin on a large scale without relying on conventional sources.

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Conflict of interest: The authors declare that there is no conflict of interest.

Author Contributions: Conception – D.D., O.S.; Design – D.D., O.S.; Materials – D.D., O.S., F.S.; Data Collection and Processing – D.D., O.S., F.S.; Analysis Interpretation – D.D., O.S., F.S.; Literature Review – D.D., O.S., F.S.; Writing – D.D., O.S., F.S.; Critical Review – O.S.

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Research Article/Araştırma Makalesi

Effects of Biochar and *Cladophora glomerata* on Wheat (*Triticum aestivum* L.) Growth and Rhizosphere Enzyme Activities

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Abstract: The positive effects of biochar on both soil quality and plant growth and also on plant growth of macroalgae have been reported in studies. Studies on biochar and macroalgae interaction are quite limited. This study was carried out according to randomized plot design in greenhouse conditions to determine the effects of biochar and *Cladophora glomerata* applications and interaction on the growth of wheat (*Triticum aestivum* L.) and some enzyme activities in the rhizosphere. Biochar and *C. glomerata* interaction increased wheat root (90%) and shoot dry weight (84.2%), root length (43.1%) and plant height (84.2%) compared to control. Biochar application increased alkaline phosphatase activity by 66.3%, while *C. glomerata* increased β-glucosidase activity by 49%. The interaction of both applications increased catalase activity by 62.1% compared to control. These findings confirm the potential of biochar and *C. glomerata* to improve wheat production by inducing growth.

Keywords: Plant growth, *Cladophora glomerata*, biochar, soil enzymes.

Biyokömür ve *Cladophora glomerata'nın* Buğday (*Triticum aestivum* L.) Gelişimi ve Rizosfer Enzim Aktivitelerine Etkileri

Öz: Biyokömürün hem toprak kalitesi hem de bitki gelişimine ayrıca makroalglerin bitki gelişimi üzerine olumlu etkileri yapılan çalışmalarda rapor edilmiştir. Biyokömür ve makroalg interaksiyonu ile ilgili çalışmalar ise oldukça sınırlıdır. Bu çalışma, biyokömür ve *Cladophora glomerata* uygulamaları ve interaksiyonunun; buğdayın (*Triticum aestivum* L.) gelişimi ve rizosferdeki bazı enzim aktiviteleri üzerindeki etkisini belirlemek için serada koşullarında tesadüf parselleri deneme desenine göre yürütülmüştür. Biyokömür ve *C. glomerata* interaksiyonu kontrolle karşılaştırıldığında buğdayın kök (%90) ve sürgün kuru ağırlığını (%84.2), kök uzunluğunu (%43.1) ve bitki boyunu (%84.2) kontrole göre artırmıştır. Biyokömür uygulaması alkalin fosfataz aktiviteyi %66.3 oranında arttırırken, *C. glomerata* β-glukosidaz altiviteyi %49 oranında artırmıştır. Her iki uygulamanın interaksiyonu katalaz aktiviteyi kontrolle karşılaştırıldığında %62.1 oranında artırmıştır. Bu bulgular, biyokömür ve *C. glomerata*'nın buğday gelişimini tetikleyerek üretimini iyileştirme potansiyelini doğrulamaktadır.

Anahtar kelimeler: Bitki gelişimi, *Cladophora glomerata*, biyokömür, toprak enzimleri.

1. Introduction

The use of organic amendments affects soil organic matter that impacts many important physicochemical and biological processes in the soil (Danish et al., 2011). Soil organic matter is an important component of the soil system and is continuously depleted, especially in semi-arid and arid regions (Diacono and Montemurro, 2010). The stability of organic matter has been attributed to the ability of carbon-containing nutrients added to soils to provide stable organic carbon (Song et al., 2020). The application of organic material can lead to a decrease in soil oxygen, increased evaporation of NH_3, immobilization of soil mineral nutrients, and the production of phytotoxic compounds (Diacono and Montemurro, 2010). In this respect, biochar provides an organic impetus to the sustainability of agriculture and the environment (Chen et al., 2020). In some cases, the incorporation of solid charred biomass (biochar) into the soil has been described to increase soil fertility and carbon stocks over long periods of time (Hu et al., 2024). Algal extracts can be used as natural plant growth stimulants in sustainable and organic plant production (Mahmoud et al., 2019). *Cladophora glomerata* is a green macroalga found in both marine and freshwater (Pikosz et al., 2016). It is considered harmful in eutrophic water reservoirs as it can increase biomass up to three times a day (Messyasz et al., 2015). Although considered an environmental problem, *C. glomerata* has been used in various applications due to the abundance of carotenoids (Michalak and Messyasz, 2021). Studies have identified the positive effect of macroalgal extracts on plant growth, including *Sargassum vulgare* (brown macroalgae) (Mahmoud et al., 2019), *Codium taylorii* (green macroalgae), and *Pterocladia capillacea* (red macroalgae) (Kassim et al., 2016). Macroalgae in the form of extracts or dry biomass were able to influence plant growth, which was particularly evidenced by the positive effect of *Ulva fasciata* (green) and *Sargassum laserifolium* (brown) on radish growth and yield (Ahmed et al., 2021)

Enzymes are important factors in the microbial activities of soil microbial communities, which are important for the biogeochemical cycle in the soil ecosystem (Aponte et al., 2020). Therefore, soil enzymes have an important role in soil organic matter transformation, nutrient release, and fertility maintenance (Goncalves-Lopes et al., 2021). By measuring soil enzyme activities, information on soil chemical properties, fertility

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levels, microbiological properties, and soil pollution status can be obtained (Jiang et al., 2021). Studies to determine the effects of biochar and *C. glomerata* on microbial parameters in soils are very limited. Therefore, in this study, the effects of biochar and *Cladophora glomerata* separately and in interaction on wheat growth and enzymatic activities of rhizosphere soil under greenhouse conditions were investigated.

2. Material and Method

2.1. Materials used in the experiment

Soil samples taken from the campus area where no previous application was made were sieved through a 2 mm sieve and filled into 3 kg pots. The soil used in the experiment has a pH of 7.2, organic matter (%) 1.22; lime (%) 2.38; EC (mmhos/cm) 1.54; N 0.14%, and has a clayey texture. *C. glomerata* used in the experiment was collected from Euphrates River in Karkamış District of Gaziantep in July 2023. Invertebrates (mussel and snail shells) in the algae were cleaned from stones and sand and dried in the shade. They were then pulverized with a Tefal Ultra High Speed Blender (shredder) at 46 000 rpm and stored in a deep freezer at -20 ◦C. Biorfe brand biochar was used as biochar. Biochar applied to the soil was added to the soil according to the instructions for use (250 g of biochar to 25 liters of soil). Wheat seeds (*Triticum aestivum* L.) were used in the experiment.

2.2. Set-up of the experiment

Wheat seeds were soaked in 10% v/v sodium hypochlorite (NaOCl) for two minutes, then washed with 70% ethanol, then with sterile distilled water, and excess water was removed on filter paper. The soil was mixed with the amount of biochar determined according to the instructions for use. *C. glomerata* was prepared at the rate of 2% in the root zone of the plant after germination and 100 ml/kg of soil was given to each pot twice with 15 days intervals. The experiment was set up as follows: a) plants grown in soil without biochar and *C. glomerata* (control); b) plants grown in soil containing biochar; c) plants grown in soil containing *C. glomerata* and without biochar; d) Plants grown in soil treated with *C. glomerata* and mixed with biochar. The experiment was conducted in three replications in a greenhouse with natural light. Plants were harvested 30 days after sowing. At the end of the harvest, plant height, green parts, root weights, and root length were determined according to Yıldız and Özgen (2004).

2.3. Determination of chlorophyll content of wheat leaves

Chlorophyll content of leaves was analyzed according to Arnon (1949). Leaf samples of each treatment were homogenized with acetone: water mixture and centrifuged at 5000x g. The absorbance of the filtrate was measured in a spectrophotometer at 645 nm and 663 nm wavelength in 3 replicates. The results were calculated in mg/l according to Arnon (1949).

2.4. Determination of some enzyme activities of rhizosphere

At the end of the harvest, soil samples adhering to the roots were taken, brought to the laboratory without waiting and analyzed. Alkaline phosphatase activity in soil was determined according to Tabatabai (1994). β-Glucosidase (EC3.3.1.21) activity was measured using p-nitrophenyl-β-D-glucopyranoside as substrate. In β-glucosidase activity, toluene was added to 0.5 g of soil sample, shaken for 15 minutes, then buffer solution and p-nitrophenyl-β-D-guloside solution were added and incubated at 37°C for 1 hour. Calcium chloride solution and tris solution (pH 12) were then added and the supernatant was measured spectrophotometrically at 410 nm after centrifugation (Yin et al., 2014). Catalase activity of rhizosphere soil was determined by Scheibler calcimeter. Phosphate buffer was added to the soil sample, H_2O_2 was placed in a glass tube, the jar was sealed with a stopper, and the oxygen output was recorded. The results were calculated as mg O_2 /5 g soil (Beck, 1971).

2.5. Statistical analysis

The results of the experiment were evaluated using the JMP11 statistical program for basic plant growth characteristics, rhizosphere catalase, alkaline phosphatase, and β-glucosidase activities.The results are given as the average of three measurements (n = 3) ± SE for each sample. Differences between treatments were determined using the Duncan Multiple Range Test.

3. Results and Discussion

3.1. Effects of applications on basic plant characteristics

The effects of *C. glomerata*, biochar treatments and the interaction of *C. glomerata* and biochar on root and shoot dry weight of wheat were examined and the results are given in Table 1. As a result of biochar and *C. glomerata* interaction, shoot wet and dry weight, root length, plant height, and root dry weight of wheat were significantly increased compared to control plants (Table 1). Co-application of *Cladophora glomerata* and biochar increased root dry weight by 78.9%, shoot dry weight by 62.8%, plant height by 26.7%, and shoot wet weight by 48.5% compared to the control.

Table 1. Effects of treatments on growth parameters of wheat

Treatments	Plant height (cm)	Root length (cm)	Shoot wet weight (g/plant)	Shoot dry weight (g/plant)	Root dry weight (g/plant)
Control	33 c*	6.07b	1.7b	0.13c	0.04c
C. glomerata (C)	44.33a	8.17a	3.28a	0.31a	0.09b
Biochar (B)	40.90b	7.77a	2.13ab	0.23b	0.07b
C+B	45a	8.67a	3.30a	0.35a	0.19a
LSD ($p<0.05$)	1.37	0.49	0.51	0.02	0.02

*Different letters in the same column are statistically significant ($p<0.05$).

Wheat plant height, root length, root and shoot weight were higher in *C. glomerata* and biochar treatments

compared to control plants. Biochar has been reported to positively affect root-associated microbial diversity producing various metabolites that promote plant growth (Jiang et al., 2021). The increase in plant growth parameters of biochar and *C. glomerata* treatments compared to the control is thought to indicate that biochar improves the potential effect of plant beneficial microorganisms on plant growth through possible stimulation of the root system. In this study, it was explained that biochar addition to soil increased plant growth as it increased nutrient availability for microbial proliferation (Khan et al., 2020). Different formulations of macroalgae have also been used to increase the production of various plants (Baroud et al., 2021; Battacharyya et al., 2016; Hamouda et al., 2022). As a result of the studies, it has been determined that macroalgae extracts contain significant amounts of mineral nutrients, carbohydrates, amino acids, osmoprotectants, and antioxidants that contribute positively to plant growth (Ali et al., 2021; Battacharyya et al., 2015; Espinosa-Anton et al., 2023). Macroalgae have also been reported to contain various mucopolysaccharides, alditols, phenols, and organic substances that increase plant productivity and conserve soil moisture (Ma et al., 2022).

The treatments increased chlorophyll content. In a study, it was reported that *C. glomerata* treatment significantly increased the chlorophyll content of radish leaves compared to the control group (Dziergowska et al., 2021). In our study, the treatments significantly increased the chlorophyll content compared to the control (Fig. 1).

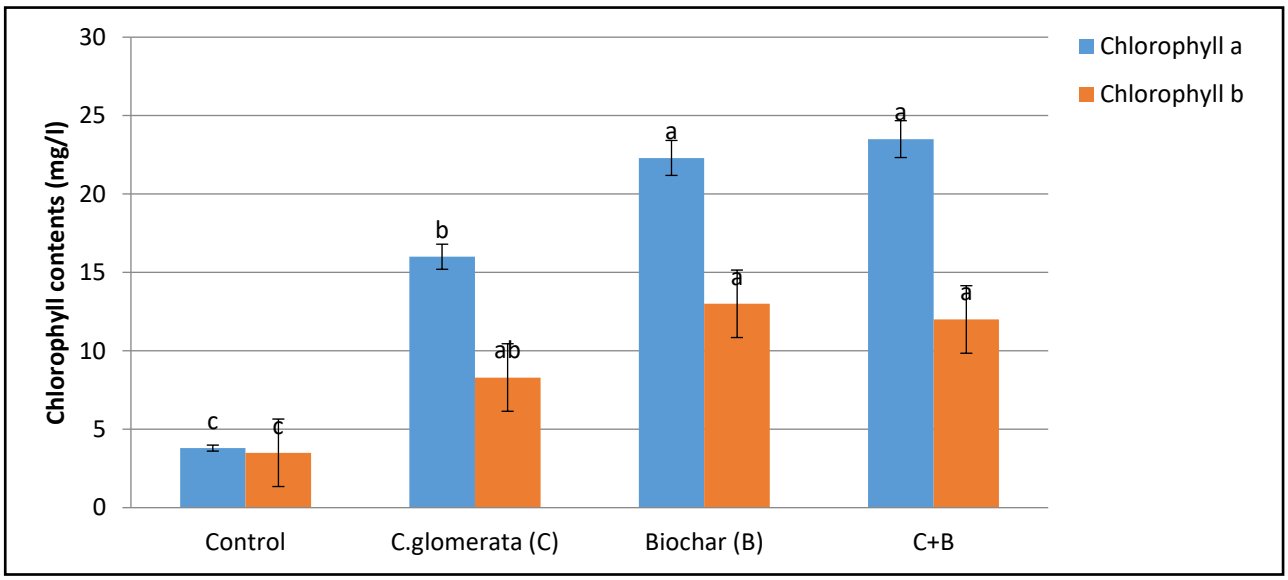

Figure 1. Effects of treatments on chlorophyll content in leaves (mg/l)

These results are similar to the increase in chlorophyll content of leaves examined in the treatment of *Codium taylorii* and *Pterocladia capillacea* extracts (Kassim et al., 2016), treatment of radish seeds with *Sargassum vulgare* extract (Mahmoud et al., 2019), *Ulva fasciata* and *Sargassum laserifolium*. The increase in chlorophyll content compared to the control may be due to the improvement in pigment biosynthesis, the content of phytohormones such as cytokinins, as well as the high concentrations of N and Mg (structural components of chlorophyll) in algal extracts. In maize plants, wheat straw biochar has been described to significantly increase plant height, chlorophyll content, water use efficiency, and grain weight due to an increase in soil P, K, N, and microbial biomass (Abbas et al., 2020). Biochar applications were found to improve pecan walnut tree height, chlorophyll content, photosynthetic rate, and N, P, Fe and K accumulation by increasing soil N content and enzyme activities (Hou et al., 2020).

3.2. Effects of applications on rhizosphere enzyme activities

Soil enzymes are important participants in the organic matter cycling and biochemical process of the soil system. Their activities are closely related to soil organic matter content, physical properties and microbial activities (Dounoras et al., 2024). Soil enzymes are one of the most active organic components in soil. They are important for the decomposition and nutrient cycling of soil material and determine soil nutrient availability and plant yield (Chen et al., 2023). It has been explained that catalase activity depends on the organic carbon content of the soil (Chen et al., 2020). In this study, it was determined that soil catalase activity increased with the addition of biochar and *C. glomerata* to the soil. As shown in Figure 2, the catalase activity of each treatment was higher than the control, indicating that the application of biochar and *C. glomerata* to the soil may be beneficial for increasing soil catalase activity. The addition of *C. glomerata* to the soil increased catalase activity by 54.2% compared to the control, while the addition of biochar increased catalase activity by 50%. In the interaction of *C. glomerata* and biochar, the activity increased by 62.1%. Our results showed that catalase activity increased in accordance with the reported results (Tu et al., 2020). The increase in catalase activities may be due to the increase in organic matter induced by the addition of biochar and *C. glomerata*, which provides sufficient substrate to promote microbial enzymatic reactions (Wang et al., 2019). This could be attributed to the coexistence of both beneficial and functional microorganisms, as nutrients and substrates in the soil were sufficient during the application period and the number of microorganisms in the soil gradually increased.

Phosphatases are produced by plants and microorganisms in soil (Chen et al., 2020). An increase in phosphatase activities was determined by adding biochar to the soil (Fig. 2). In terms of biochar addition, it is reported that the available carbon sources contained in biochar work together to accelerate microorganism growth in soil (Khan et al., 2019); thus, biochar promotes enzyme activities. Biochar plays an important role in maintaining microbial growth as well as providing energy (Paz-Ferreiro et al., 2013). The addition of biochar significantly increased the alkaline phosphatase activity of the soil as it brought active substances with it. The addition of biochar and *C. glomerata* to the soil increased the alkaline phosphatase activity compared to the control group (Fig. 3).

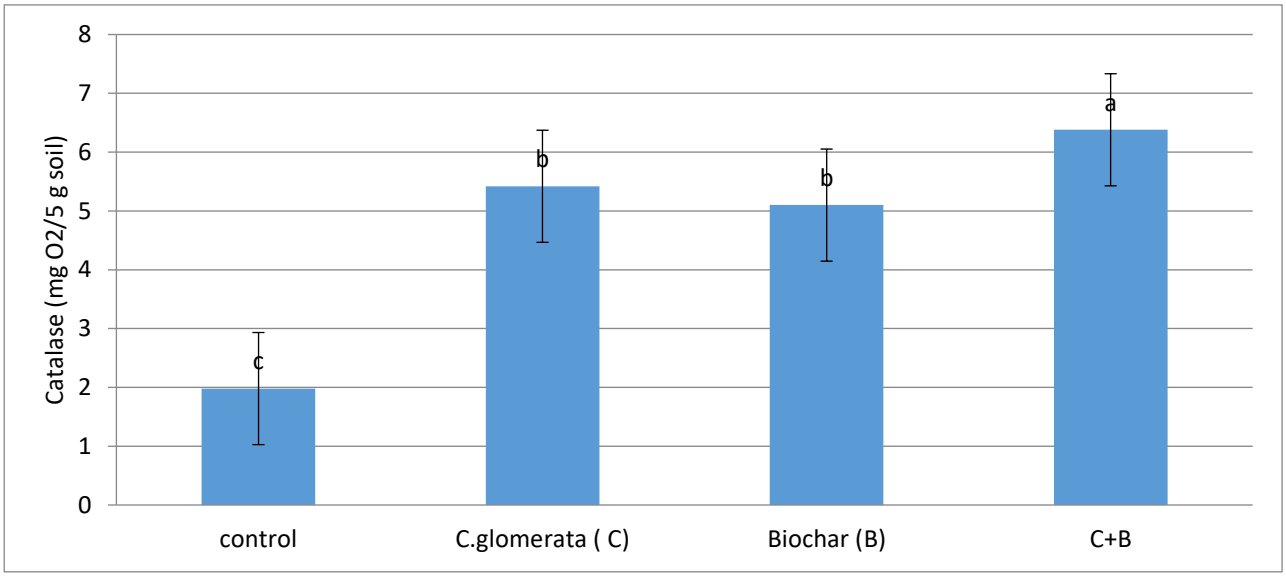

Figure 2. Effects of treatments on catalase enzyme activity of rhizosphere soil. The difference between means given with the same letter is not significant.

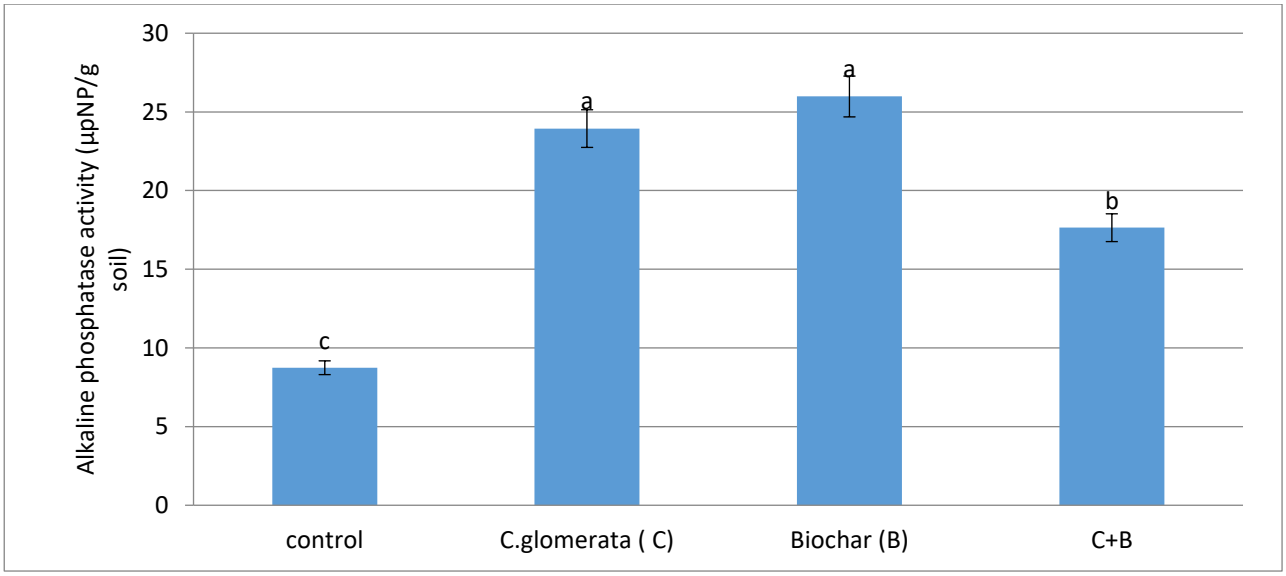

Figure 3. Effects of treatments on alkaline phosphatase enzyme activity of rhizosphere soil. The difference between means given with the same letter is not significant.

β-Glucosidase, urease, phosphatase, and sucrase are important for the conversion of soil nutrients. Among them, sucrase and glucosidase are associated with carbon conversion in soil and phosphatase can increase the availability of phosphorus. Agricultural management measures such as fertilization and irrigation can significantly affect soil enzyme activities (Chen et al., 2023). Our study shows that the β-glucosidase activity of *C. glomerata*-treated soil significantly increased compared to non-*C. glomerata*-treated soil (Fig. 4). The increase in enzyme activity promotes nutrient cycling and improves soil nutrient levels. It has been described that macroalgae can reduce nitrogen leaching and ammonia volatilization in soil and improve nitrogen use efficiency (Chen et al., 2023). As a plant-derived preparation, macroalgae are mainly composed of organic carbohydrates such as polysaccharides. Since *C. glomerata* is thought to be able to improve soil properties and microflora, it can increase soil enzyme activities and soil enzymes can actively participate in nutrient cycling, providing mineral nutrients for plants (Chen et al., 2023). The application of biochar in this study affected the activity rate of the β-glucosidase enzyme,

which may be related to the increase in carbon availability promoted by biochar. β-glucosidase actively participates in the carbon cycle and is involved in the hydrolysis of organic products (Khan et al., 2019). Moreover, the presence of volatile compounds in biochar may have contributed to the enzyme activity (Liao et al., 2016). In this study, the findings of increased β-glucosidase activity due to the inclusion of biochar and *C. glomerata* in soil compared to the control are supported by previous studies (Ali et al., 2019, Song et al., 2020). The increase in β-glycosidase activity in soil may have contributed to the increase in the activity of other enzymes. β-glycosidase has been reported to release low molecular weight sugars, which are energy sources for microorganisms in soil, leading to increased microbial activity (Pathan et al., 2017).

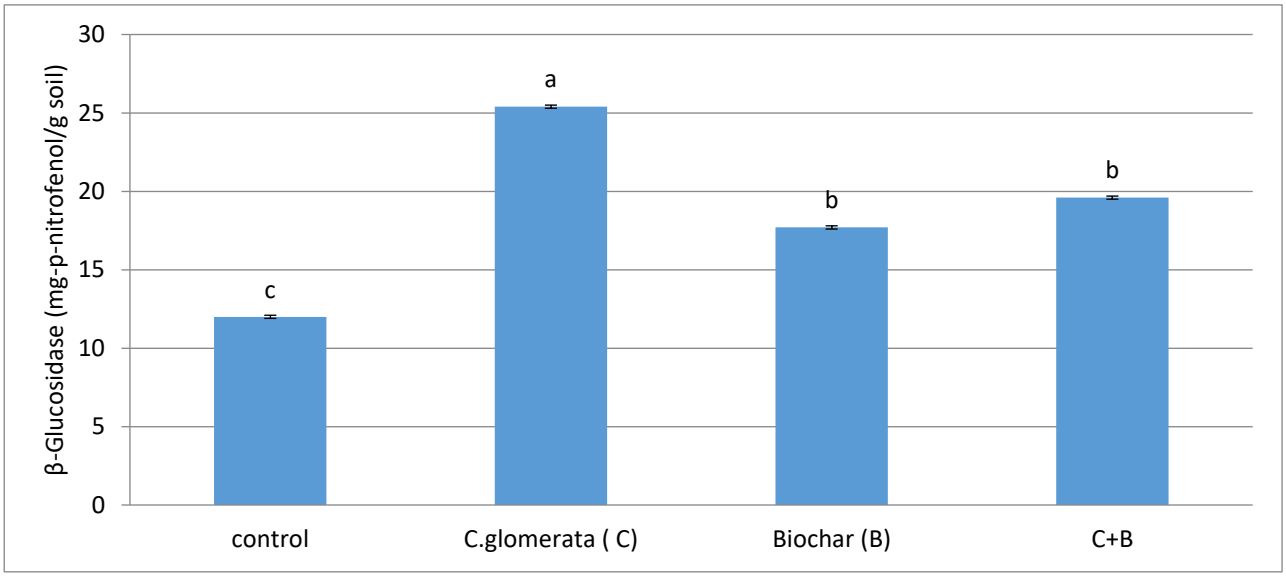

Figure 4. Effects of treatments on β-Glucosidase enzyme activity of rhizosphere soil. The difference between means given with the same letter is not significant.

Soil enzymes are indicators of soil quality as they are directly related to soil microbial activity and biogeochemical cycling of nutrients (Jiang et al., 2021). An increase in the activity of extracellular enzymes (β-glucosidase, β-xylosidase and β-d-cellobiosidase) involved in the sulfur and carbon cycle in soil has been recorded in biochar application (Jiang et al., 2021). Several studies (Goncalves-Lopes et al., 2021; Jiang et al., 2021; Tu et al., 2020) also revealed that biochar showed different effects on enzyme activities in different soil types. The application of macroalgae products in sufficient quantities has been described to indirectly affect the nutrient uptake capacity of plants by improving the physical, chemical, and biological properties of the soil or substrate (Ma et al., 2022). *Ulva ohnoi* was found to contain polyanionic compounds with strong chelating activity, such as phenolics and ulvan, which can form complexes with metal ions essential for plant nutrition (Illera-Vines et al., 2020). Similarly, amino acids (e.g., cysteine, glycine, histidine, and glutamic acid) contained in some macroalgae species have been found to bind to some trace elements to form very small and electrically neutral chelates, accelerating the uptake and transport of elements important in plant nutrition (Ma et al., 2022). Macroalgae applications have also been found to have positive effects on nutrient cycling and plant roots by promoting the growth of beneficial root-associated microorganisms (Popko et al., 2018). Metabolites produced by algae are used by microorganisms in soils as sources of nutrients and carbon, leading to an increase in the microbial population in the soil, and some microorganisms are capable of producing extracellular enzymes to hydrolyze organic P in soil that cannot be used by plants, which is thought to increase the efficient use of phosphorus by plants (Higo et al., 2020). However, further studies are needed to investigate the long-term effects of *C. glomerata* and biochar on soil microorganisms that benefit the soil in various ways and to reveal their ecological role in biochar-treated soils.

4. Conclusions

Biochar and *C.glomerata* treatments significantly increased the activities of rhizosphere enzymes. This may be due to the proliferation of beneficial microorganisms that enhance microorganism activities and enzymatic reactions. This study also showed that the addition of biochar and *C. glomerata* to the soil significantly improved phosphatase, catalase, and β-glucosidase activities compared to the control. The treatments were also found to have an effect on the main plant growth parameters and the results showed a synergistic effect of *C. glomerata* and biochar on plant growth and soil enzymes. These findings provide new insights into the potential of biochar and *C. glomerata* co-application on both plant growth and soil enzyme activities to improve wheat production. Our study demonstrated that biochar and soil-applied *C. glomerata* can be used as a sustainable amendment to enhance plant growth and improve soil quality, which will play a vital role in agriculture.

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Conflict of interest: The authors declare that there is no conflict of interest.

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Research Article/Araştırma Makalesi

Search for Aflatoxin M1 in Raw Milk and Butter Samples

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Abstract: In this study, the presence of aflatoxin M1 (AFM1) in raw milk of cows, sheep, goats, buffalo, and cow butter offered for consumption in some settlements in Turkey and Iraq in the Spring of 2022 was investigated and, taking into account legal regulations, it was determined whether it poses a danger to human health. Toxin contents in milk were compared between these sampling points. In the study, 50 raw cow milk, 60 raw sheep milk, 30 raw goat milk, 30 raw buffalo milk, and 40 butter samples were collected and AFM1 presence was analyzed by ELISA method. It was observed that the AFM1 level in the dark and goat milk samples collected from Ankara was statistically significantly higher than those collected from Mosul but did not exceed the European Union and Turkish Food Codex (TFC) limits (50 ng/kg). AFM1 levels in buffalo milk and cow butter sampled from Mosul were observed to be statistically significantly higher than those sampled in Kayseri and Ankara, respectively, and exceeded the TFC limits. It was determined that the AFM1 level of cow milk samples from Ankara and Mosul exceeded the TFC limit. As a result, since the presence of AFM1 detected in buffalo milk, cow milk, and cow butter samples collected from Mosul and cow milk samples collected from Ankara is important for public health, agricultural products used as feed in these regions should be selected correctly, stored under appropriate conditions, routinely analyzed for aflatoxin, and strictly inspected. Furthermore, the organization of training programs on good agricultural practices aimed at educating livestock breeders and milk and dairy product producers and raising producer and consumer awareness are believed to safeguard public health.

Keywords: Mycotoxin, milk, butter, ELISA.

Çiğ Süt ve Tereyağı Örneklerinde Aflatoksin M1 Aranması

Öz: Bu çalışmada, 2022 ilkbahar mevsiminde, Türkiye ve Irak'taki bazı yerleşim yerlerinde tüketime sunulan inek, koyun, keçi, manda çiğ sütlerinde ve inek tereyağında aflatoksin M1 (AFM1) varlığı araştırıldı ve yasal mevzuatlar dikkate alınarak insan sağlığı yönünden tehlike oluşturup oluşturmadığı belirlendi. Sütlerdeki toksin içerikleri, bu örnekleme noktaları arasında karşılaştırıldı. Çalışmada, 50 adet çiğ inek sütü, 60 adet çiğ koyun sütü, 30 adet çiğ keçi sütü, 30 adet çiğ manda sütü ve 40 adet tereyağı örneği toplanarak AFM1 varlığı ELISA yöntemi ile analiz edildi. Ankara'dan toplanan koyu ve keçi sütü örneklerindeki AFM1 düzeyinin, Musul'dan toplananlara göre istatistiksel olarak önemli düzeyde yüksek olduğu, ancak Avrupa Birliği ve Türk Gıda Kodeksi (TGK) limitini (50 ng/kg) aşmadığı görüldü. Musul'dan örneklenen manda sütü ve inek tereyağındaki AFM1 düzeylerinin, sırasıyla Kayseri ve Ankara'da örneklenenlere göre istatistiksel olarak önemli düzeyde yüksek olduğu ve TGK limitlerini aştığı gözlendi. İnek sütü örneklerinden ise Ankara ve Musul'dan örneklenenlerin AFM1 düzeyinin TGK limitini aştığı tespit edildi. Sonuç olarak, Musul'dan toplanan manda sütü, inek sütü ve inek tereyağı ile Ankara'dan toplanan inek sütü örneklerinde tespit edilen AFM1 varlığının halk sağlığı açısından önemli olması nedeniyle, bu bölgelerde, yem olarak kullanılan tarımsal ürünlerin doğru seçimi, uygun koşullarda muhafaza edilmesi, rutin olarak aflatoksin yönünden analiz edilmesi ve sıkı bir şekilde denetlenmesi gerekmektedir. Ayrıca, besi yetiştiriciliği yapan kişiler ile süt ve süt ürünleri üreticilerinin bilgilendirilmesine yönelik iyi tarım uygulamaları konusunda eğitim programları düzenlenerek üretici ve tüketici bilincinin arttırılması sonucu halk sağlığının korunacağı düşünülmektedir.

Anahtar kelimeler: Mikotoksin, süt, tereyağı, ELISA.

1. Giriş

Süt, yaşamın ilk yıllarından itibaren insan sağlığının gelişmesi ve korunması için gerekli birçok besin maddesini içeren, besleyiciliği yüksek bir besindir. Bilimsel araştırmalar, sütün aynı zamanda çevresel ve gıda kirleticilerinin taşıyıcısı olabileceğini, bunların da sağlık üzerindeki olası olumsuz etkilerini göstermiştir. Sütte bulunan mikroorganizmalar ve metabolitleri, doğum yapan memelilerin sıvılarına ve dokularına geçerek sağlığa zarar verebilmektedir (De Souza vd., 2021). İnsan ve hayvan tüketimine yönelik tarım ürünleri, mikotoksinler adı verilen ikincil toksik mantar metabolitleri ile kontamine olabilir. Kirlenme, hasattan önce veya hasat sırasında veya uygun şekilde depolanmadıklarında (yani yetersiz sıcaklık ve nem koşulları) meydana gelebilir. Hayvan yemlerinde oluşan mikotoksinler, insan tüketimi için gıda üretiminde kullanılan hayvan dokularına veya sıvılarına ulaşabilmektedir. Kirlenmiş gıda ve yemler, insanlarda ve hayvanlarda çeşitli sağlık sorunlarına ve ekonomik kayıplara neden olabilir. Aynı gıdada farklı mikotoksinlerin aynı anda ortaya çıkması muhtemeldir. Bunun nedeni, bir tür mantarın birkaç mikotoksin üretebilmesi ve sıklıkla istila edilmiş bir substratın çeşitli küf türleri içermesidir. Tek bir üründe birkaç mikotoksinin birlikte ortaya çıkması durumunda, katkı maddesi veya

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sinerjik toksik etkiler beklenebilir (Flores-Flores vd., 2017). Aflatoksinler, hayvanlar ve insanlar üzerindeki kanserojen ve hepatotoksik etkileri nedeniyle en önemli mikotoksinler olarak kabul edilir. Aflatoksinler başta *Aspergillus flavus*, *Aspergillus nomius* ve *Aspergillus parasiticus* türlerine ait olanlar olmak üzere Aspergillus cinsindeki bazı mantar türleri tarafından sentezlenen toksik, ikincil metabolitlerdir (De Souza vd., 2021). Çeşitli aflatoksin türleri arasında, gıda maddelerinin doğal kontaminantları olarak en sık rastlananları aflatoksin B1 (AFB1), B2 (AFB2), G1 (AFG1) ve G2 (AFG2)'dir. AFB1 en yüksek toksisiteye sahipken, bu toksin ayrıca Uluslararası Kanser Araştırmaları Ajansı tarafından 1. grup kanserojen olarak sınıflandırılmaktadır. Ayrıca AFM1 ve AFM2 sırasıyla AFB1 ve AFB2'nin hepatik biyotransformasyonu ile üretilir ve hayvanlardan idrar ve süt yoluyla atılabilir (De Souza vd., 2021). AFM1, süt ve süt ürünlerinde de standarttan daha yüksek tespit edilmiştir. Bu, birçok gelişmekte olan ülke insanının aflatoksin kontaminasyonu tehlikesiyle karşı karşıya olduğunu göstermektedir (Negash, 2018). Aflatoksinlerin gıdalara kontaminasyonu; direkt, indirekt ve taşınma olmak üzere 3 şekilde gerçekleşmektedir. Direkt kontaminasyon, gıdada mikotoksin üreten küfün gelişmesi ve mikotoksin oluşturmasıyla gerçekleşir. İndirekt kontaminasyon ise hammaddelere veya yardımcı maddelere mikotoksin bulaşması ve bunların gıda üretiminde kullanılmasıyla meydana gelmektedir. Taşınma (kalıntı-carry over) ile kontaminasyon (röle kontaminasyonu), AFB1 bulaşmış yemlerle beslenen laktasyon dönemindeki hayvanların vücutlarına aldıkları bu toksinleri metabolize ederek AFM1 şeklinde süte geçmesiyle meydana gelmektedir (Karaoğlan vd., 2022). Süt ve süt ürünleri, AFM1 gibi sağlığı tehdit eden elementleri insan diyetine sokma potansiyeline sahiptir (Mehenktaş, 2019). Toksin, AFB1'in ilk alımından 12-24 saat sonra sütte tespit edilebilir. AFB1 uygulaması durdurulduğunda, sütteki AFM1 konsantrasyonu 72 saat sonra tespit edilecek seviyeden düşer (Rahimi vd., 2010). Aflatoksinler, akut zehirlenme (aflatoksikoz), hepatosellüler karsinom, çocuklarda büyüme bozukluğu ve bağışıklık baskılamadan sorumludur.

AFM1, süt ve süt ürünlerinde nispeten stabildir. Çünkü yüksek sıcaklıklarda pastörizasyon ve sterilizasyon gibi işleme prosedürleriyle veya tuz ilavesi ile yok edilemez. Aflatoksinlerin sağlığı tehdit edici etkileri nedeniyle, birçok gelişmiş ülke gıda maddelerinde maksimum AFM1 konsantrasyonları belirlerken, çoğu gelişmekte olan ülke hala ABD Gıda ve İlaç İdaresi (FDA) ve Avrupa dahil olmak üzere düzenleyici kurumlar tarafından belirlenen yasal kısıtlamalara, izin verilen maksimum AFM1 seviyelerine güvenmektedir (Özkan & Onmaz, 2019; Ghaffarian Bahraman vd., 2020). Avrupa Topluluğuna (Avrupa Komisyonu) ve Codex Alimentarius Komisyonuna (CAC) göre, süt ve süt ürünlerinde maksimum AFM1 seviyesi 50 ng/kg'ı geçmemelidir (Karimi Dehcheshmeh vd., 2021). Aflatoksin kontaminasyonunun yaygın olarak görülmesi ve zararlı etkileri nedeniyle sütte aflatoksin M1'in saptanmasına ve ölçülmesine ihtiyaç vardır. Bu çalışma, Türkiye ve Irak'tan toplanan çiğ süt ve tereyağı örneklerinde AFM1 varlığının ve miktarının saptanması ve Türk Gıda Kodeksi tarafından belirlenen limit değere (50 ng/kg) uygunluğunun belirlenmesi amacıyla tasarlanmıştır. Bu çalışmanın sonuçları, iki farklı ülkede önemli ekonomik değere sahip olan ve bolca tüketilen bu süt ürünlerinin insan sağlığı yönünden tehlike oluşturup oluşturmadığı konusunda literatüre katkı sağlamaktadır. Ayrıca bu çalışma, örnekleme yaptığımız bölgelerdeki üreticilere çiğ süt ve tereyağı üretimine yönelik bazı tedbirlerin alınması yönünde bir farkındalık oluşturma niteliğindedir. Bu farkındalık, bundan sonraki üretim yöntemlerinde halk sağlığının korunması adına mevcut durumun iyileştirilmesi yönünde bazı adımların atılmasına zemin hazırlayacaktır.

2. Materyal ve Metot

2.1. Süt Örneklerinin Temini

Bu çalışmada, 2022 yılı ilkbahar döneminde, 60 adet çiğ koyun sütü, 50 adet çiğ inek sütü, 30 adet çiğ manda sütü, 30 adet çiğ keçi sütü ve 40 adet inek tereyağı örneği, Türkiye (Ankara, Bursa, Kayseri) ve Irak'ta (Musul) bulunan mandıralardan temin edildi. Süt (50 ml) ve tereyağı (100 g) örnekleri, steril kapaklı falkon tüpler içinde, +4°C'lik soğuk zincirde laboratuvara getirildi ve -17°C'de buzdolabında muhafaza edilerek en kısa sürede analiz edildi.

2.2. Süt Örneklerinin Aflatoksin M1 Analizi

Süt örneklerinde AFM1 varlığı ve düzeyi kompetetif ELISA (Enzyme-Linked Immunosorbent Assay) yöntemi ile ROMER LABS tarafından verilen prosedüre göre AgraQuant® Aflatoksin M1, High Sensitivity 10002120 Elisa test kiti kullanılarak tespit edildi. Kullanılan test kitinin ölçme limiti 5 ppt ve geri alma oranı süt için ortalama %95'tir. Sonuçların değerlendirilmesi, ROMER LABS tarafından hazırlanan "Spreadsheet" adlı bilgisayar paket programı kullanılarak yapıldı. Bu paket programının değerlendirme prensibi şu şekildedir: Standart ve örnekler için elde edilen absorbans değerlerinin ortalama değerleri, sıfır standardın absorbans değeri ile bölünür ve sıfır standart, 1'e eşit hale getirilir (Tablo 1). Standartlar için hesaplanan değerler, semilogaritmik grafik kağıdı üzerine ng/L (ppt) cinsinden AFM1 konsantrasyonuna karşı koordinatlar sistemine girilerek standart eğri ve bu standart eğriye ait denklem elde edilir. Bu denklem üzerinden analiz edilen her bir örneğin AFM1 konsantrasyonu ng/L cinsinden hesaplanır. Homojen olarak karıştırılmış her bir süt örneğinden 5 ml süt alınarak 10°C'de, 3500 devirde, 10 dakika süreyle santrifüje edildikten sonra tüpün üstündeki yağ tabakası pastör pipeti ile çekilerek alındı. Yağı alınmış bu süt testte direkt olarak kullanıldı (İşleyici vd., 2012).

2.3. Tereyağı Örneklerinin Aftaloksin M1 Analizi

ELISA'nın gıdalarda aflatoksin M1 analizi için güvenilir bir yöntem olduğu rapor edilmiştir (Türkoğlu, 2018; Merve & Ocak, 2019). Çalışmada, analiz edilen tereyağı örneklerinin ekstraksiyon prosedürü kullanılan test kitindeki (ROMER LABSAgraQuant® Aflatoksin M1, High Sensitivity 10002120) üretici firma talimatlarına göre gerçekleştirildi. Çalışma kapsamında, ilgili mandıralardan çiğ sütten elde edilmiş tereyağları kullanıldı. 37°C'ye ayarlanmış su banyosunda (Nüve®, BM401, Türkiye) eritilen 5 gr yağ örneğine 25 mL (%70'lik) metanol ilave edilerek 40°C sıcak su banyosunda, 10 dakika inkübe edildi. Daha sonra, karışım oda sıcaklığında yavaşça 10

dakika karıştırılarak ekstrakte edildi. Eksrakt, filtre kağıdından (Whatman No:1, 125 mm) süzüldü ve 5 mL süzülmüş çözeltiye 15 mL damıtılmış su ve 0.25 mL Tween-20 ilavesinden sonra 2 dakika homojenize edildi. Homojenizasyon işleminden sonra, bu karışım tekrar filtre edildi (Whatman No:1, 125 mm) ve elde edilen özütün 100 µL'si AFM1 analizi için testte kullanıldı. Örneklerin analiz edilmesi ve sonuçların değerlendirilmesi için standart solüsyonlar (0, 5, 10, 25, 50 ve 100 ppt konsantrasyonda AFM1 içeren solüsyonlar) ve hazırlanan süt örnekleri için yeterli sayıda kuyucuk hesaplandı. Standart solüsyonların ve hazırlanan örneklerin her birinden otomatik pipet ile 100 µL alınarak kuyucuklara aktarıldı ve oda ısısında (20-25°C) ve karanlık ortamda 45 dakika bekletildi. Daha sonra kuyucuklardaki sıvı boşaltılıp, kuyucuklara 8 uçlu otomatik pipet yardımıyla 300 µL yıkama solüsyonu eklenerek beş defa yıkandı. Yıkanan her bir kuyucuğa 8 uçlu otomatik pipet yardımıyla 100 µL enzim konjugat solüsyonu ilave edildi ve tekrar oda ısısında (20-25°C) ve karanlıkta 15 dakika bekletildikten sonra 96 kuyucuklu plaka yıkama solüsyonu ile beş defa yıkandı. Her bir kuyucuğa sırayla 100 µL substrat 8 uçlu pipet yardımıyla pipetlendikten sonra nazikçe karıştırıldı ve 15 dakika oda ısısında ve karanlıkta bekletildi. Son olarak her bir kuyucuğa 100 µL durdurma solüsyonu ilave edilerek iyice karıştırıldı ve ELISA okuyucuda (Thermo Scientific™ Multiskan™ FC Microplate) 450 nm'de 60 dakika içinde okutularak sonuçlar RomerLabsSpreadsheet ile değerlendirildi (Azam vd., 2021; Matabaro vd., 2017). Elde edilen sonuçlar, Avrupa Birliği ve Türk Gıda Kondeksi tarafından belirlenen limit değeri (50 ng/kg) ile kıyaslandı.

2.4. İstatistiksel Analizler

Analizler sonucunda farklı örnekleme noktalarından elde dilen veriler arasında istatistiksel olarak önemli düzeyde bir ilişki olup olmadığını ortaya koymak için varyans analizi (Tukey) ve t testinden yararlanıldı (İşleyici et al., 2012). Analizlerde SPSS 24.0 paket programı kullanıldı.

3. Bulgular

Örnekleme noktalarından temin edilen ve analizi yapılan çiğ koyun sütü örneklerinde AFM1 konsantrasyonu 1.07-20.62 ng/L arasında bulundu. ELISA analiz sonuçlarına göre 60 çiğ koyun sütü örneğinin tamamı çiğ süt tebliğine uygunluk arz etmektedir. Analiz sonuçlarına göre 50 çiğ inek sütü örneğinden 34 tanesindeki AFM1 konsantrasyonu 5-50 ng/L aralığında bulundu. Bu konsantrasyonlar, TGK (<50 ng/L, TGK maksimum limit değeri) maksimum limit değerinin altında olması nedeniyle çiğ süt tebliğine uygunluk arz etmektedir. 50 çiğ inek sütü örneğinden 16 tanesindeki AFM1 konsantrasyonunun ise TGK maksimum limitin üzerinde çıktığı tespit edildi. Manda sütü örneklerinde, AFM1 miktarları 5.50-95.60 ng/L aralığında bulundu. Analiz sonuçlarına göre, 30 çiğ manda sütü örneğinden 20 tanesi, AFM1 konsantrasyonu maksimum limit değerin altında olması nedeniyle çiğ süt tebliğine uygunluk arz etmektedir. 50 çiğ manda sütü örneğinden 10 tanesindeki AFM1 konsantrasyonu ise maksimum limitin üzerinde bulundu. Çiğ keçi sütü örneklerinde AFM1 miktarları 1.31-14.99 ng/L aralığında tespit edildi. Buna göre, 30 çiğ keçi sütü örneğinin tamamı çiğ süt tebliğine uygunluk arz etmektedir. İnek tereyağı örneklerinde AFM1 miktarları 5.76-51.75 ng/L aralığında bulundu. Analiz sonuçlarına göre, 40 inek tereyağı örneğinden 32 tanesi çiğ süt tebliğine uygunluk arz etmektedir. 8 örnekteki AFM1 konsantrasyonu ise maksimum limitin üzerinde çıkmıştır.

Bölgeler arası kıyaslama yapılacak olursa; TR-Ankara-Ayaş örnekleme noktasından elde edilen koyun sütü örneklerindeki AFM1 düzeyinin TR-Ankara-Elmadağ-Hasanoğlan noktaları ve Irak'tan alınanlara göre istatistiksel olarak önemli düzeyde yüksek olduğu tespit edildi ($p<0.001$). Koyun çiğ süt örneklerinin toplandığı Irak'taki örnekleme noktaları (Telefer, Musul, Karakoyun Köyü ve Aljazira) arasında ise istatistiksel olarak önemli bir fark görülmedi ($p>0.05$) (Tablo 2.a ve Şekil 1). TR-Ankara-Elmadağ-Hasanoğlan örnekleme noktasından elde edilen inek sütü örneklerindeki AFM1 düzeyinin diğer örnekleme noktalarından alınan inek sütü örneklerindeki AFM1 düzeyine göre istatistiksel olarak önemli düzeyde yüksek olduğu tespit edildi ($p<0.001$). Ayrıca, TR-Ankara-Mamak-Bayındır ve IR-Musul-Karakoyun Köyü örnekleme noktalarından alınan inek sütü örneklerindeki AFM1 düzeylerinin, TR-Ankara-Bala ve IR-Telefer örnekleme noktalarından alınanlara göre önemli düzeyde yüksek olduğu istatistiksel olarak ($p<0.001$) belirlenirken, Ankara-Mamak-Bayındır ve IR-Musul-Karakoyun Köyü örnekleme noktaları arasında AFM1 düzeyleri bakımından istatistiksel olarak önemli bir fark görülmedi ($p>0.05$). Bununla birlikte, TR-Ankara-Bala ve IR-Telefer örnekleme noktaları arasında da AFM1 düzeyleri bakımından istatistiksel olarak önemli bir fark tespit edilmedi ($p>0.05$) (Tablo 2.a ve Şekil 2). Irak'taki tek örnekleme noktası olan IR-Musul'dan elde edilen manda sütü örneklerindeki AFM1 düzeyinin diğer örnekleme noktalarından alınanlara göre istatistiksel olarak önemli düzeyde yüksek olduğu tespit edildi ($p<0.001$). TR-Bursa örnekleme noktasından elde edilen manda sütü örneklerindeki AFM1 düzeyinin TR-Kayseri örnekleme noktasından elde edilenlere göre istatistiksel olarak önemli düzeyde yüksek olduğu tespit edildi ($p<0.01$) (Tablo 2.b ve Şekil 3). TR-Ankara-Ayaş örnekleme noktasından elde edilen keçi sütü örneklerindeki AFM1 düzeyinin diğer örnekleme noktalarından alınan keçi sütü örneklerindeki AFM1 düzeyine göre istatistiksel olarak önemli düzeyde yüksek olduğu tespit edildi ($p<0.001$). Bununla birlikte, IR-Telefer örnekleme noktasından toplanan keçi sütü örneklerindeki AFM1 düzeyinin, istatistiksel olarak TR-Ankara-Ayaş örnekleme noktasına göre önemli düzeyde düşük ($p<0.001$), IR-Musul örnekleme noktasından toplanan keçi sütü örneklerindeki AFM1 düzeyine göre yüksek olduğu belirlendi ($p<0.001$) (Tablo 2.b ve Şekil 4). IR-Musul-Karakoyun Köyü örnekleme noktasından temin edilen inek tereyağı örneklerindeki AFM1 düzeyinin diğer örnekleme noktalarından alınanlara göre istatistiksel olarak önemli düzeyde yüksek olduğu tespit edildi ($p<0.001$). Bunula birlikte, diğer örnekleme noktaları olan TR-Ankara-Ayaş, TR-Ankara-Elmadağ-Akçaali ve TR-Ankara-Mamak-Bayındır'dan toplanan inek tereyağı örneklerindeki AFM1 düzeyleri arasında istatistiksel olarak önemli bir fark tespit edilmedi ($p>0.05$) (Tablo 2.c ve Şekil 5).

Tablo 1.AFM1 analizinde kullanılan standartların absorbans değerlerine göre elde edilen referans aralıkları.

Table 1. Reference ranges obtained according to absorbance values of the standards used in AFM1 analysis.

Standart	Standart Konsantrasyonları (ng/L)	Ortalama Absorbans Değeri	Log Konsantrasyonları	B/Bo
1	0	0.786		1.000
2	5	0.577	0.699	0.734
3	10	0.475	1.000	0.605
4	25	0.344	1.398	0.437
5	50	0.245	1.699	0.311
6	100	0.147	2.000	0.188

Tablo 2.a. Çiğ koyun ve inek süt örneklerinde aflatoksin M1 konsantrasyonu ve dağılımı.

Table 2.a. Concentration and distribution of aflatoxin M1 in raw sheep and cow milk samples.

			Konsantrasyon AFM1 (ng L^{-1})			Örneklerdeki AFM1 dağılımı/(%) Örnek Tipi				Pozitif	Limiti Aşan
Analiz Edilen Örnek	Ülke	Örnekleme Noktaları	Ortalama	Min.	Maks.	TE	< 5	5-50	> 50	n	n
Koyun Sütü (n=60)	Türkiye (n=20)	Ankara-Ayaş	11.23±1.40	6.40	20.62	-	-	10 (%100)	-	10 (%100)	-
		Ankara-Elmadağ-Hasanoğlan Köyü	6.74±0.47	4.28	10.09	-	1 (%10)	9 (%90)	-	10 (%100)	-
		TÜRKİYE	8.98±0.88	4.28	20.62	-	1 (%5)	19 (%95)	-	20 (%100)	-
	Irak (n=40)	Telefer	2.65±0.29	1.52	3.76	-	10 (%100)	-	-	10 (%100)	-
		Musul	2.29±0.32	1.07	3.78	-	10 (%100)	-	-	10 (%100)	-
		Musul-Karakoyun Köyü	3.08±0.13	2.63	3.65	-	10 (%100)	-	-	10 (%100)	-
		Musul-Aljazira	3.63±0.17	2.60	4.70	-	10 (%100)	-	-	10 (%100)	-
		IRAK	2.91±0.14	1.07	4.70	-	40 (%100)	-	-	40 (%100)	-
		TOPLAM	4.94±0.48	1.07	20.62	-	41 (%68)	19 (%32)	-	60 (%100)	-
İnek Sütü (n=50)	Türkiye (n=30)	Ankara-Bala	15.23±0.49	12.88	17.42	-	-	10 (%100)	-	10 (%100)	-
		Ankara-Elmadağ-Hasanoğlan Köyü	70.44±3.20	53.58	86.71	-	-	-	10 (%100)	10 (%100)	10 (%100)
		Ankara-Mamak-Bayındır	43.01±1.31	38.08	51.08	-	-	9 (%90)	1 (%10)	10 (%100)	1 (%10)
		TÜRKİYE	42.89±4.33	12.88	86.71	-	-	19 (%63)	11 (%37)	30 (%100)	11 (%37)
	Irak (n=20)	Musul-Karakoyun Köyü	50.41±3.08	38.30	65.89	-	-	5 (%50)	5 (%50)	10 (%100)	5 (%50)
		Telefer	8.40±0.69	5.30	12.40	-	-	10 (%100)	-	10 (%100)	
		IRAK	29.40±5.06	5.30	65.89	-	-	15 (%75)	5 (%25)	20 (%100)	5 (%50)
		TOPLAM	37.50±3.39	5.30	86.71	-	-	34 (%68)	16(%32)	50 (%100)	16(%32)

Tablo 2.b. Çiğ manda ve keçi süt örneklerinde aflatoksin M1 konsantrasyonu ve dağılımı.

Table 2.b. Concentration and distribution of aflatoxin M1 in raw buffalo and goat milk samples

			Konsantrasyon AFM1 (ng L^{-1})			Örneklerdeki AFM1 dağılımı/(%) Örnek Tipi				Pozitif	Limiti Aşan
Analiz Edilen Örnek	Ülke	Örnekleme Noktaları	Ortalama	Min.	Maks.	TE	< 5	5-50	> 50	n	n
Manda Sütü (n=30)	Türkiye (n=20)	Kayseri	6.44±0.64	5.50	7.18	-	-	10 (%100)	-	10 (%100)	-
		Bursa	8.94±2.00	7.34	12.34	-	-	10 (%100)	-	10 (%100)	-
		TÜRKİYE	7.69±1.94	5.50	12.34	-	-	20 (%100)	-	20 (%100)	-
	Irak (n=10)	Musul	91.19±2.80	87.78	95.60	-	-	-	10 (%100)	10 (%100)	10 (%100)
		IRAK	91.19±2.80	87.78	95.60	-	-	-	10 (%100)	10 (%100)	10 (%100)
		TOPLAM	35.52±7.32	5.50	95.60	-	-	20 (%67)	10 (%33)	30 (%100)	10 (%33)
Keçi Sütü (n=30)	Türkiye (n=10)	Ankara-Ayaş	12.72±0.44	11.09	14.99	-	-	10 (%100)	-	10 (%100)	-
		TÜRKİYE	12.72±0.44	11.09	14.99	-	-	10 (%100)	-	10 (%100)	-
	Irak (n=20)	Musul	1.94±0.12	1.31	2.57	-	10 (%100)	-	-	10 (%100)	-
		Telefer	6.01±0.76	3.52	8.48	-	5 (%50)	5 (%50)	-	10 (%100)	-
		IRAK	3.98±0.60	1.31	8.48	-	15(%75)	5 (%25)	-	20 (%100)	-
		TOPLAM	6.89±0.87	1.31	14.99	-	15 (%50)	15 (%50)	-	30 (%100)	-

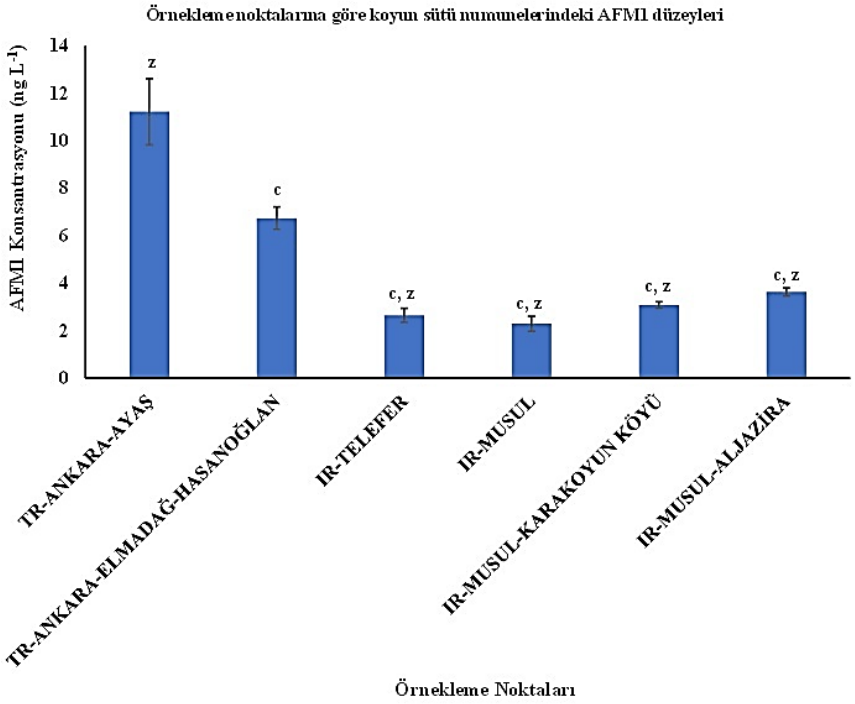
Tablo 2.c. İnek tereyağı örneklerinde aflatoksin M1 konsantrasyonu ve dağılımı.

Table 2.c. Concentration and distribution of aflatoxin M1 in cow butter samples.

			Konsantrasyon AFM1 (ng L^{-1})			**Örneklerdeki AFM1 dağılımı/(%) Örnek Tipi**				**Pozitif**	**Limiti Aşan**
Analiz Edilen Örnek	**Ülke**	**Örnekleme Noktaları**	**Ortalama**	**Min.**	**Maks.**	**TE**	**< 5**	**5-50**	**> 50**	**n**	**n**
İnek Tereyağı (n=40)	**Türkiye (n=30)**	Ankara-Ayaş	16.36±3.65	5.76	44.41	-	-	10 (%100)	-	10 (%100)	-
		Ankara-Elmadağ-Akçaali	23.30±2.30	14.02	34.04	-	-	10 (%100)	-	10 (%100)	-
		Ankara-Mamak-Bayındır	17.15±2.29	7.10	27.15	-	-	10 (%100)	-	10 (%100)	-
		TÜRKİYE	***18.94±1.67***	***4.28***	***20.62***	**-**	**-**	***30 (%100)***	**-**	***30 (%100)***	**-**
	Irak (n=10)	Musul-Karakoyun Köyü	50.65±0.24	49.60	51.75	-	-	2 (%20)	8(%80)	10 (%100)	**8 (%80)**
		IRAK	***50.65±0.24***	***49.60***	***51.75***	**-**	**-**	***2 (%20)***	***8 (%80)***	***40 (%100)***	***8 (%80)***
		TOPLAM	***26.86±2.53***	***5.76***	***51.75***	**-**	**-**	***32 (%80)***	***8 (%20)***	***40 (%100)***	***8 (%20)***

Şekil 1. Örnekleme noktalarına göre koyun sütü numunelerindeki AFM1 düzeyleri

Figure 1. AFM1 levels in sheep milk samples, according to sampling points

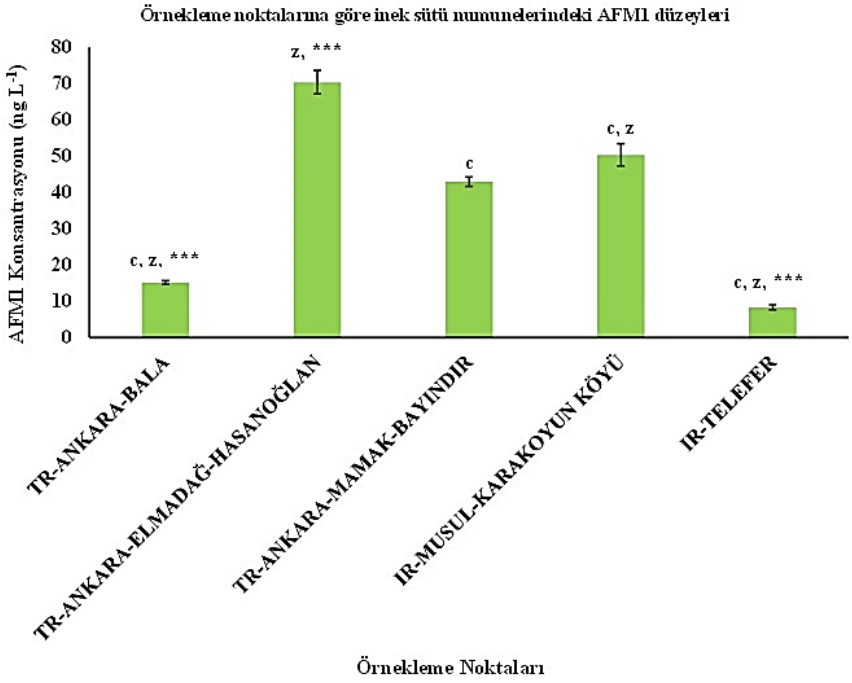


TR-Ankara-Ayaş örnekleme noktasına göre karşılaştırma; **a**: p<0.05, **b**: p<0.01, **c**: p<0.001

TR-Ankara-Elamadağ-Hasanoğlan örnekleme noktasına göre karşılaştırma; **x**: p<0.05, **y**: p<0.01, **z**: p<0.001

Şekil 2: Örnekleme noktalarına göre inek sütü numunelerindeki AFM1 düzeyleri

Figure 2: AFM1 levels in cow milk samples, according to sampling points



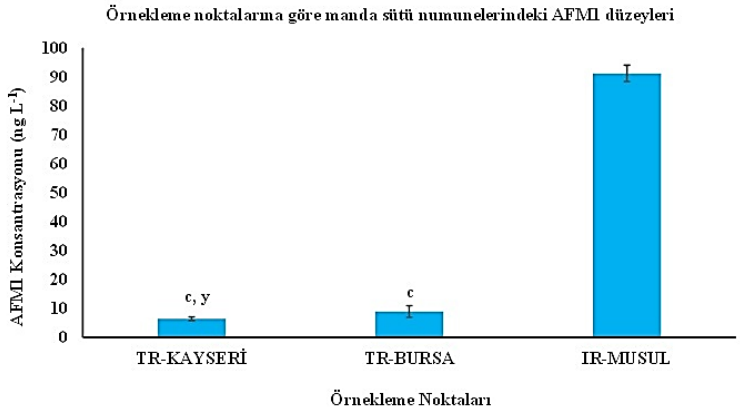
TR-Ankara-Elmadağ-Hasanoğlan örnekleme noktasına göre karşılaştırma; **a**: p<0.05, **b**: p<0.01, **c**: p<0.001

TR-Ankara-Mamak-Bayındır örnekleme noktasına göre karşılaştırma; **x**: p<0.05, **y**: p<0.01, **z**: p<0.001

IR-Musul-Karakoyun Köyü örnekleme noktasına göre karşılaştırma; *: p<0.05, **: p<0.01, ***: p<0.001

Şekil 3: Örnekleme noktalarına göre manda sütü numunelerindeki AFM1 düzeyleri

Figure 3: AFM1 levels in buffalo milk samples, according to sampling points

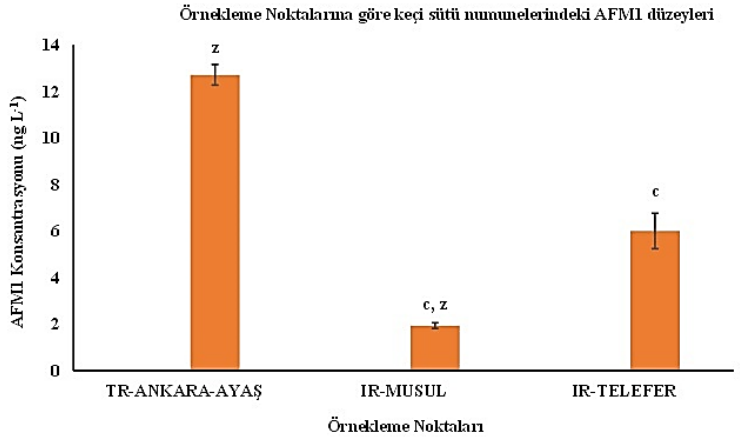


IR-Musul örnekleme noktasına göre karşılaştırma; a: p<0.05, b: p<0.01, c: p<0.001

TR-Bursa örnekleme noktasına göre karşılaştırma; x: p<0.05, y: p<0.01, z: p<0.001

Şekil 4: Örnekleme noktalarına göre keçi sütü numunelerindeki AFM1 düzeyleri

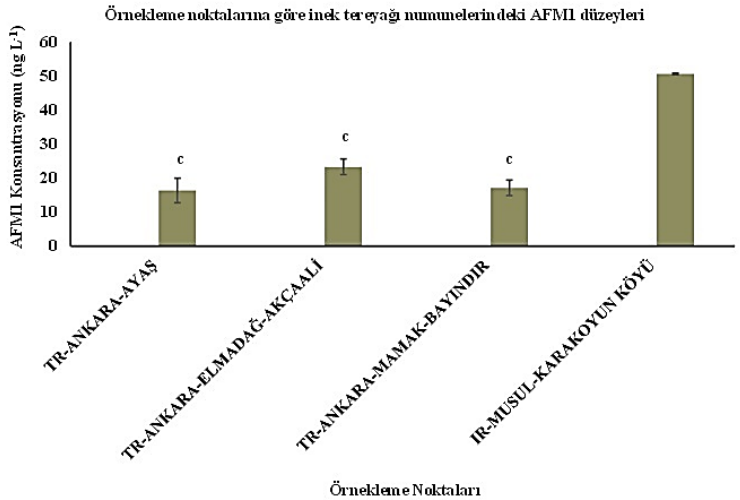
Figure 4: AFM1 levels in goat milk samples, according to sampling points



TR-Ankara-Ayaş örnekleme noktasına göre karşılaştırma; **a**: p<0.05, **b**: p<0.01, **c**: p<0.001
IR-Telefer örnekleme noktasına göre karşılaştırma; **x**: p<0.05, **y**: p<0.01, **z**: p<0.001

Şekil 5: Örnekleme noktalarına göre inek tereyağı numunelerindeki AFM1 düzeyleri

Figure 5: AFM1 levels in cow butter samples, by sampling points



IR-Musul-Karakoyun Köyü örnekleme noktasına göre karşılaştırma; **a**: p<0.05, **b**: p<0.01, **c**: p<0.001

4. Tartışma

Süt ve süt ürünlerinde bulunan AFM1, insan sağlığı için önemli riskler oluşturmaktadır (İşleyici vd., 2012). Bunun için yasal düzenlemeler belirlenmiş veya tavsiye edilmiştir. Bu düzenlemeler, ekonomik koşullara bağlı olarak bir ülkeden diğerine farklılık göstermektedir (Nile vd., 2016).

Bu çalışmada, Türkiye ve Irak'taki bazı yerleşim yerlerinden toplanan çiğ süt (koyun, inek, manda, keçi sütü) ve tereyağı (inek tereyağı) örneklerinde aflatoksin M1 varlığı test edildi. Çalışmada toplanan koyun (60 örnek), inek (50 örnek), manda (30 örnek) ve keçi (30 örnek) çiğ süt örneklerinin tamamının aflatoksin M1 yönünden pozitif olduğu bulundu. Çalışma sonuçlarına benzer olarak literatürde birçok araştırmacı da (Karadal vd., 2018; Bahrami vd., 2016; Thukral vd., 2022; Tomašević vd., 2015; Gide vd., 2020; Mahmoudi, 2014; Özkan vd., 2019, Iqbal vd., 2011) analiz ettikleri çiğ süt örneklerinin aflatoksin M1 yönünden pozitif olduğunu rapor etmiştir. Çalışmamızda, Ankara-Elmadağ-Hasanoğlan köyü, Ankara-Mamak-Bayındır ve Musul-Karakoyun Köyü'nden örneklenen inek sütlerinin %32'si TGK limitini aşmıştır. Kayseri-Bursa-Musul'dan toplanan manda sütü örneklerinin %33'ü, tereyağı örneklerinin ise %20'si TGK limitlerini aşmıştır (Tablo 2a ve 2b). Çalışmamıza benzer olarak, Türkiye'de Iğdır (Yurt & Uluçay, 2017) ve Kayseri (Buldu vd., 2011) illerinden toplanan inek sütü örneklerinin sırasıyla %80 ve %70'inde, AFM1 miktarının TGK limitini aştığı tespit edilmiştir. Diğer ülkelerde yapılan çalışmalardan örnek verecek olursak; çiğ süt örneklerinde AFM1 oranları, Sudan'da %95.45 (Elzupir & Elhussein, 2010), Nijerya, Yobe Eyalet Üniversitesi Damaturu çiftliğinde %80 (Gide vd., 2020), Suriye'de %59 (Ghanem & Orfi, 2009), Hindistan'ın Pencap bölgesinde %56.2 (Thukral vd., 2022) olarak tespit edilmiştir. Bu sonuçlar çalışmamızda bulunan oranlardan daha yüksektir. Bazı çalışmalarda ise bizim tespit ettiğimizden daha düşük oranlarda TGK limitini aşan AFM1 varlığı tespit edilmiştir. Örneğin Niğde ilinde örneklerin sadece %10'unun (Karadal vd., 2018), İran'da %15.4' ünün (Fallah vd., 2016), Pakistan'da %16.3' ünün (Iqbal vd., 2011), Orta Hırvatistan'da %1.87'sinin (Bilandžić vd., 2022) limit değerleri aştığı tespit edilmiştir. Bu farklılıklar muhtemelen ineklerin tükettiği yem maddelerindeki AFB1 miktarına bağlı olarak değişkenlik göstermektedir. AFB1, %50-%60 arasında çevresel nem koşulları altında ve %13-%18 arasında nem içeren yemlerde kolaylıkla gelişebilen bazı küfler tarafından üretildiği için hasat öncesi ve hasat sırasında yerel hava koşulları ve depolama koşullarına dikkat edilmelidir (Hashemi, 2016). Manda sütü örnekleri ile ilgili çalışmamızda, Irak-Musul'dan alınan örneklerde AFM1 düzeyinin AB ve TGK limitlerini aştığı görüldü. Bu sonuçlar, Irak-Musul'da yaygın olarak tüketilen süt veya süt ürünü örneklerinin AFM1 kontaminasyonu için sürekli gözetiminin önemini göstermiştir.

Çiğ sütte AFM1 seviyesi, mevsimlere göre değişkenlik göstermektedir. Mevsimler arası karşılaştırma çalışmalarında, hayvanların çiğ sütündeki AFM1 seviyesinin, ot, çim ve kaba yemlerin daha bol olduğu ve meralardan daha fazla beslenilen ilkbahar ve yaz aylarında, konsantre yemle beslenilen kış aylarına göre daha düşük olduğu tespit edilmiştir. Hayvanları yaz sonuna kadar yeşil ve taze otlarla beslemek, yemdeki AFB1 miktarının azalmasına ve dolayısıyla çiğ sütteki AFM1 seviyesinin düşmesine neden olmaktadır (Roila vd., 2021; İşleyici, 2015). Koyun sütü ve keçi sütü örneklerimizde limiti aşan bulunmamıştır. Bu yüzden Irak ve Türkiye'de koyun ve keçi sütü örneklerimiz TGK'ya göre tüketim için uygundur ve insan sağlığını etkilememektedir. Bu sonuçların sebeplerinden biri, yukarıda belirttiğimiz gibi, süt numunelerini, hayvanların en sağlıklı beslendiği ilkbahar mevsiminde temin etmiş olmamızdır.

Çalışmamızda, tereyağı örneklerinden Irak-Musul'dan temin edilenlerin %80'inin AB ve TGK limitlerini aştığı, Ankara'dan toplananların ise limiti aşmadığı görüldü. Genelde, tereyağı diğer süt ürünlerinden daha az aflatoksin içermektedir. Çünkü tereyağı suda çözünür ve kazein proteini içerir. AFM1 ile kontamine olmuş kremadan tereyağı üretimi sırasında mikotoksinlerin çoğunun yıkama suyuna geçtiği bildirilmiştir (Özkan & Onmaz, 2019; Ráduly vd., 2020; Agriopoulou vd., 2020). Çalışmamızda Ankara'dan toplanan tereyağı örneklerinde AFM1 düzeyi her ne kadar TGK limitini aşmadıysa da bazı numunelerde yüksekti. Bunun ana sebebi ise, tereyağının elde edildiği inek sütü örneklerinde yüksek miktarda AFM1'e rastlanmasıdır. Bu yüzden bu bölgelerde inek sütü için önlemler alınmalı ve AFM1 seviyelerini en az miktara düşürmek hedeflenmelidir. AFM1'i kontrol etmek için, hayvansal kullanım amaçlı tarımsal ürünlerde küf gelişimini ve aflatoksin B1 (AFB1) oluşumunu önleyerek süt hayvanlarının beslenmelerinde AFB1 kontaminasyonunu azaltmak gerekmektedir. Yem eldesinde kullanılan silaj gibi ürünler AFB1 açısından en fazla kontaminasyona uğrayan yem ham maddelerindendir. Silaj üretimi sırasında aflatoksin oluşumu çoğunlukla hasat zamanı, döllenme, sulama, haşere kontrolü, silaj nemi ve depolama uygulamaları gibi faktörlerden etkilenmektedir. Bu nedenle muhafaza neminin, çekirdek mekaniksel hasarının, tahıl temizleme uygulamalarının ve muhafaza sıcaklığının dikkatle kontrol edilmesi gerekmektedir (Aksoy ve Sezer, 2019).

5. Sonuç

Çalışmamızda TR-Ankara-Elmadağ-Hasanoğlan inek sütü numunelerindeki AFM1 düzeyinin yüksek seviyeye ulaştığı ve TGK limitini aştığı bulundu. Tespit edilen AFM1 varlığı halk sağlığı açısından önemlidir ve sistematik olarak kontrol edilmesi gerekmektedir. Bu nedenle, Türkiye'de inek sütünde AFM1 aranması çalışmalarının daha fazla yapılmasını önermekteyiz. Aynı zamanda IR-Musul manda sütü, inek sütü ve inek tereyağı örneklerindeki AFM1 düzeyinin de AB ve TGK limitini aştığı görüldü. Irak'ta bu tür AFM1 aranması çalışmaları çok eksik veya yok denebilecek kadar azdır. Bu yüzden bu bölgede de bu tür çalışmaların başlatılması ve sürdürülmesi önerilmektedir. Gerekli koruyucu önlemlerin çiftlikten sofraya kadar her aşamada alınması gerektiği dikkate alındığında, aflatoksinlerin neden olduğu potansiyel sağlık problemleri ve sonuçları konusunda besi yetiştiriciliği yapan kişiler ile süt ve süt ürünleri üreticilerinin bilgilendirilmelerine yönelik eğitim programlarına ağırlık verilmelidir. Hayvanlara verilen yemlerden kaynaklanabileceği ihtimali de düşünülerek, AFM1 kontaminasyonunu süt ve süt ürünlerinde minimize etmek için yem hammaddelerinin seçimi,

hayvan yemlerinin rutin olarak aflatoksin yönünden (AFB1) kalite kontrolünün analiz edilmesi, modern üretim tekniklerinin yaygınlaştırılması, süt hayvanlarının beslenmesinde kullanılan yem maddelerinin depolanma koşullarının iyileştirilmesi ve sıkı bir şekilde denetlenmesi önem arz etmektedir. İyi tarım uygulamalarının hayata geçirilmesi, üretici ve tüketici bilincinin arttırılması ile halk sağlığı korunacaktır. Çalışmamızın sonuçları, çiğ süt ve tereyağı örneklerinde, ileriki zamanlarda yapılacak olan toksikolojik ve biyokimyasal analizler için bir ön bilgi niteliğindedir.

Teşekkür: Bu çalışma Karabük Üniversitesi, Bilimsel Araştırma Projeleri birimi tarafından KBÜBAP-22YL-021 numaralı proje ile maddi olarak desteklenmiştir.

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Research Article/Araştırma Makalesi

Palynological Investigation of Some *Trifolium* L. (Fabaceae) Species Distributed in Şanlıurfa

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Abstract: In this study, the pollen morphology of 5 species (*Trifolium boissieri* Guss. ex-Soy. -Will. & Godr., *T. dasyurum* C.Presl, *T. pauciflorum* d'Urv., *T. scabrum* L., and *T. spumosum* L.) belonging to the genus *Trifolium* L. from the Fabaceae family, distributed in Şanlıurfa province, was examined with light and electron microscopes. Within the framework of the palynological study, pollen shapes, ornamentations, pore and colpus lengths and widths were determined and their measurements were given. Pollen grains are generally monad in structure, have trizonocolporate aperture, and subprolate to prolate-spheroidal shape. Ornamentation generally shows significant differences in polar and equatorial regions. In the pollen grains examined, perforate, psilate-perforate, and reticulate ornamentation is seen in the polar region, while microreticulate and reticulate ornamentation is dominant in the equatorial region. While the polar axis lengths were determined to be between 38.67-29.19 µm on average, the equatorial axis length was determined to be between 33.79-23.45 µm on average. As a result of the study, it was determined that characters such as pollen shape, pollen size, and surface ornamentation differed among species and these were the characters that could have taxonomic value in the systematic distinction of species.

Keywords: Pollen, morphology, palynology, Türkiye.

Şanlıurfa'da Yayılış Gösteren Bazı *Trifolium* L. (Fabaceae) Türlerinin Palinolojik Yönden İncelenmesi

Öz: Bu çalışmada, Şanlıurfa ilinde yayılış gösteren Fabaceae familyasından *Trifolium* L. cinsine ait 5 türün (*Trifolium boissieri* Guss. ex Soy.-Will. & Godr., *T. dasyurum* C.Presl, *T. pauciflorum* d'Urv., *T. scabrum* L., *T. spumosum* L.,) polen morfolojisi ışık ve elektron mikroskopları ile incelenmiştir. Palinolojik çalışma çerçevesinde polen şekilleri, polen yüzey ornamentasyonları, por ve kolpus uzunlukları ve genişlikleri tespit edilmiş ve ölçümleri verilmiştir. Polenler genel monad yapıda, trizonokolporat apertüre sahip ve subprolat ile prolat-sferoidal şekle sahiptir. Ornamentasyon ise genellikle polar ve ekvatoral bölgede belirgin farklılıklar göstermektedir. İncelenen polenlerde polar bölgede perforat, psilat-perforat ve retükalat ornamentasyon görülürken Ekvatoral bölgede ise mikroretikülat ve retikülat ornamentasyon baskındır. Polar eksen uzunlukları ortalama 38,67- 29,19 µm arasında tespit edilirken ekvatoral eksen uzunluğu ise ortalama 33,79- 23,45 µm arasında tespit edilmiştir. Polen şekli, polen büyüklüğü ve yüzey ornamentasyon gibi karakterlerin türler arasında farklılık gösterdiği ve türlerin sistematik ayrımında taksonomik değere sahip karakterler olduğu tespit edilmiştir.

Anahtar kelimeler: Polen, morfoloji, palinoloji, Türkiye.

1. Introduction

The flora of Türkiye is among the world's leading flora in terms of species richness. There are 167 families, 1320 genera, and a total of 11707 taxa belonging to these genera in Turkey. The Fabaceae family is the world's third-largest family of seed plants, with 770 genera and approximately 20,000 species. Approximately 1228 taxa belonging to 72 genera are distributed in Türkiye, making it the country's second-largest family in species number. The number of endemic Fabaceae taxa in Türkiye is 383 (Güner et al., 2012; Christenhusz & Byng, 2017).

Trifolium L. (Fabaceae), which exhibits the highest species richness in the Mediterranean basin, is represented by approximately 300 species in the world, while in Türkiye, it is represented by 107 species and 167 taxa. Fourteen of these taxa are determined to be endemic to Türkiye. The genus *Trifolium* is divided into seven sections (Güner et al., 2012; Keskin et al., 2023).

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The genus is known by local names such as "Üçgül" or "Yonca" among the public. Since the genus *Trifolium* is also important as meadow plants for fattening and dairy animals, it is one of the economically valuable genera in the Fabaceae family (Güner et al., 2012; Keskin et al., 2023).

The genus *Trifolium* is not easy to classify in classical sense due to the richness of its species. For this reason, important characteristics such as pollen, chromosomes, and genetic characteristics are frequently used in genus systematics (Keskin, 2001a, 2001b; Güner et al., 2012). Palynological and seed morphology studies are frequently used in the classification of the Fabaceae family (Pınar et al., 2009; Çeter et al., 2012; İşgör et al., 2012; Çeter et al., 2013a; 2013b; Kahraman et al., 2013; Pınar et al., 2014; Karaman et al., 2017; Metin et al., 2018; Khan et al., 2019; Altın et al., 2021; Bapir & Galalaey, 2023; Liao et al., 2021; Taşlıyurt et al., 2023; Altın et al., 2024;). Pollen grains of taxa belonging to the genus *Trifolium* also show variations among species. *Trifolium* pollen grains generally have different shapes,

from perprolate to spheroidal. Apertures can be of different types such as tricolporate, tricolpate or polycolpate. Ornamentation and pollen size also show significant variations among species (Gazar, 2003; Taia, 2004; Koçyiğit et al., 2013; Öztürk, 2013; Akçin et al., 2017).

In this study, a palynological study was carried out on some species of the genus *Trifolium* distributed in Şanlıurfa. Pollens belonging to the species were examined under light microscope (LM) and scanning electron microscope (SEM) to determine pollen morphology.

2. Material and Method

The study material consists of *Trifolium boissieri*, *T. dasyurum*, *T. scabrium*, *T. spumosum*, and *T. paucuflorum* species distributed in Şanlıurfa (Table 1). The collected species were identified with the help of the work called Flora of Turkey (Davis, 1965-1988; Davis et al., 1988). The samples were kept at Harran University Herbarium (HARRAN).

2.1. Palynological Method

2.1.1. Light Microscope Method

Pollen morphology studies using light microscopy were performed according to the Wodehouse (1935) method. Pollen samples were examined and photographed using a light microscope equipped with a Leica DM3000 Digital Imaging System using 100x immersion objective. For each analyzed character, measurements were made from 20 pollen samples and the average was taken. Pollen equatorial (E) and polar axis lengths (P), exine and intine thicknesses, colpus length (Clg) and width (Clt), and pore length (Plg) and width (Plt) were measured. Measurements were performed using the AlaMet, S.0.06 program.

2.1.2. Scanning electron microscope (SEM) method

Pollen samples were placed on aluminum staples using double-sided adhesive tape. Pollen samples were coated with gold using a Cressington Sputter Coater device, and then microphotographs were taken using a Quanta FEG 250 model Scanning Electron Microscope (SEM). Pollen surface morphology and ornamentation analysis of taxa were performed using SEM microphotographs and relevant literature (Ertdman, 1969; Faegri & Iversen, 1975; Punt et al., 2007; Hesse et al., 2009).

The morphological features of the pollen grains were determined by taking into account pollen shape, polar axis, equatorial axis, exine and intine thickness, ornamentation, colpus length, colpus width, and colpus ornamentation characters.

3. Results

The pollen grains examined were generally found to have monad, trizonocolporate aperture structure, and subprolate and prolate-spheroidal shapes. Ornamentation generally showed significant differences in the polar and equatorial areas. Perforate, psilate-perforate, and reticulate ornamentation were seen in the polar area, while microreticulate and reticulate ornamentation were dominant in the equatorial area. While the polar axis lengths were determined to be between 38.67 and 29.19 on average, the equatorial axis length was determined to be between 33.79 and 23.45 on average (Table 2).

3.1. *Trifolium boissieri*

Pollen grains were radially symmetric, isopolar, and trizonocolporate. The polar axis was 29.19 µm and the equatorial axis was 23.45 µm. The P/E ratio was calculated as 1.26. According to this ratio, the pollen shape was subprolate. In polar appearance, it was circular. Ornamentation was perforated in the polar area and around the aperture and microreticulate in the equatorial area. Exine thickness was 0.77 µm, and intine thickness was 0.35 µm. Colpus is thin, long (Clg 17.41 µm, Clt 2.5 µm) and operculate. The operculum membrane had psilate ornamentation. The pore was lolongate, Plg was 6 µm, and Plt was 4.83 µm (Fig. 1).

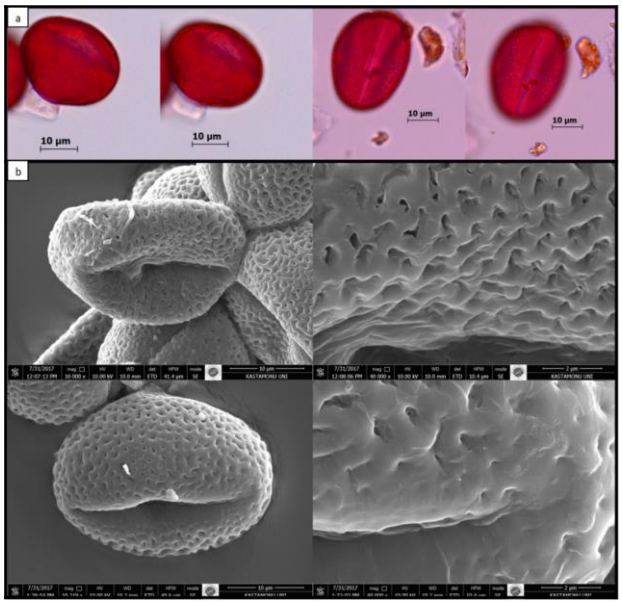

Figure 1. a. Light microscope photographs of *T. boissieri*, b. SEM photographs

3.2. *Trifolium dasyurum*

Pollen grains were radially symmetric, isopolar, and trizonocolporate. The polar axis was 38.67 µm, and the equatorial axis was 33.79 µm. The P/E ratio was calculated as 1.07. According to this ratio, the pollen shape was determined as prolate-spheroidal. In the polar view, it was circular-semi triangular. Ornamentation was determined as psilate around the aperture and reticulate in polar and equatorial areas. Exine thickness was determined as 0.87 µm and intine thickness was determined as 0.53 µm. Colpus was thin, long (Clg 28.41 µm, Clt 4.5 µm) and operculate. Operculum membrane had psilate ornamentation. Pores were longitudinally extended and had a suboblate shape. Plg was determined as 12.41 µm and Plt as 11.58 µm (Fig. 2).

3.3. *Trifolium scabrum*

Pollen grains were radially symmetric, isopolar, and trisonocolporate. The polar axis was 38.45 µm and the equatorial axis was 29.10 µm. The P/E ratio was calculated as 1.32. According to this ratio, the pollen shape was subprolate. In polar view, it was semi-triangular. Ornamentation was determined as psilate-perforate around the aperture, and as microreticulate in the polar region and equatorial region. Exine thickness was 0.78 µm, and intine thickness was 0.43 µm. Colpus was thin, long (Clg 25.09 µm, Clt 2.48 µm), and operculate. Operculum

membrane had granulate ornamentation. The pore was longitudinally extended and had a suboblate shape. Plg was determined as 5.99 µm and Plt as 7.47 µm (Fig. 3).

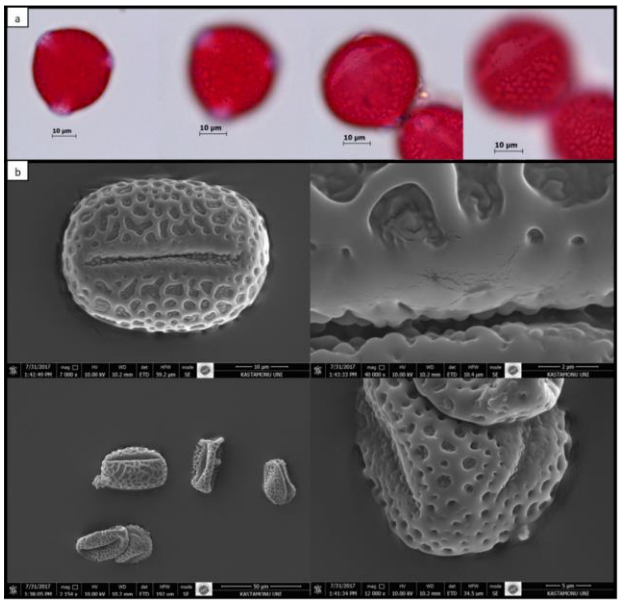

Figure 2. a. Light microscope photographs of *T. dasyurum*, b. SEM photographs

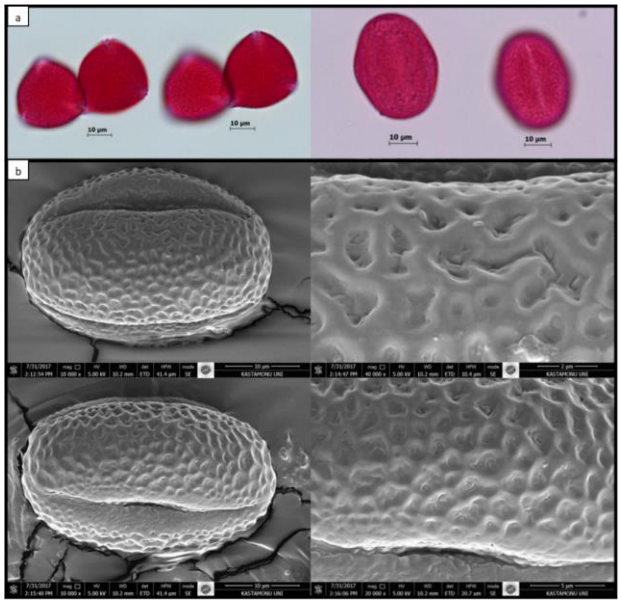

Figure. 3. a. Light microscope photographs of *T. scabrum*, b. SEM photographs

3.4. *Trifolium spumosum*

Pollen grains were radially symmetric, isopolar, and trizonocolporate. The polar axis was 33.25 µm and the equatorial axis was 27.05 µm. The P/E ratio was calculated as 1.22. According to this ratio, the pollen shape was subprolate. In polar view, it was circular in shape. Ornamentation was determined as psilate-perforate around the aperture and as reticulate in the polar and equatorial area. Exine thickness was 0.72 µm and intine thickness was 0.41 µm. Colpus was thin, long (Clg 26.43 µm, Clt 3.10 µm), and operculate. The operculum membrane had granulate ornamentation. The pore was longitudinally extended and had a prolate-spheroidal shape. Plg was determined as 8.28 µm and Plt as 7.53 µm (Fig. 4).

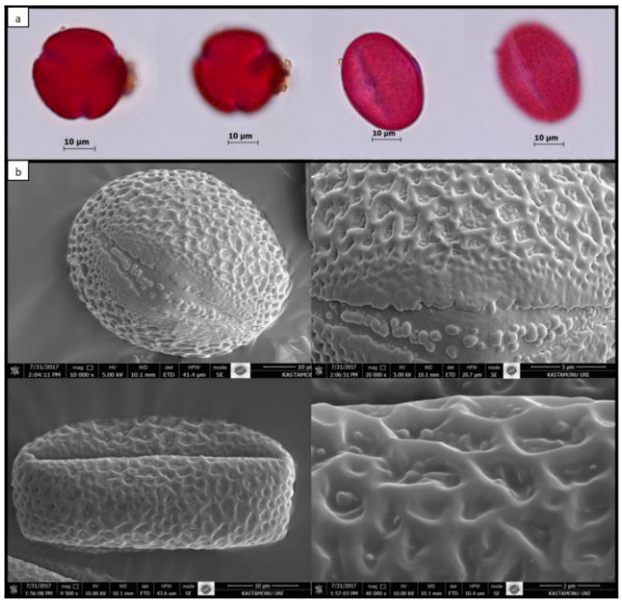

Figure 4. a. Light microscope photographs of *T. spumosum*, b. SEM photographs

3.5. *Trifolium pauciflorum*

Pollen grains were radially symmetric, isopolar, and trizonocolporate. The polar axis was determined as 33.84 µm and the equatorial axis as 29.74 µm. The P/E ratio was calculated as 1.13. According to this ratio, the pollen shape was determined as prolate spheroidal. In polar appearance, it was circular. Ornamentation was determined as perforate around aperture and microreticulate in polar equatorial area. Exine thickness was determined as 0.53 µm and intine thickness was determined as 0.28 µm. Colpus was thin, long (Clg 25.8 µm, Clt 3.22 µm), and operculate. Operculum membrane had granulate ornamentation. Pore was longitudinally extended and had subprolate shape. Plg was determined as 11.75 µm and Plt was determined as 9.52 µm (Fig. 5).

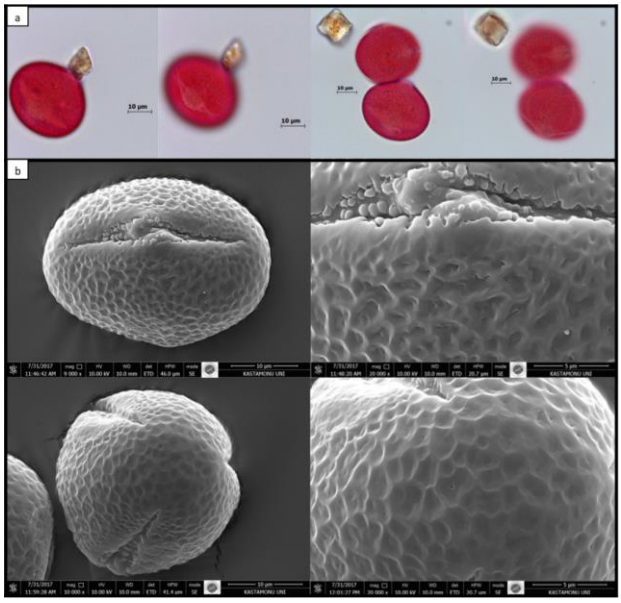

Figure 5. a. Light microscope photographs of *T. pauciflorum*, b. SEM photographs

Table 1. Locality where the samples were collected

Species	Locality	Flowering Period
T. boissieri	Şanlıurfa-Hilvan 10th km, Karaköprü location, 550 m, roadside	April-May
T. spumosum	Şanlıurfa-Bozova road: Around Kızlar village, 720 m, field edge, Şanlıurfa-Hilvan highway 10th km, Karaköprü location, 550 m, roadside, Şanlıurfa-Hilvan highway 32nd km, opposite the airport, 700 m, roadside	April-May
T. scabrum	Şanlıurfa-Hilvan highway 10th km, Karaköprü location,	April-May
T. dasyurum	Şanlıurfa-Viranşehir karayolu 45. km, 665 m, step-kayalık, Şanlıurfa-Viranşehir, step-kayalık, Şanlıurfa - Viranşehir 43. km,	April-May
T. pauciflorum	Şanlıurfa: Center, Osmanbey campus, 500 m, step, Şanlıurfa-Bozova road: Tektaş village junction, 720 m, field edge, Şanlıurfa-Hilvan highway 10 km, Karaköprü location, Şanlıurfa-Viranşehir 45 km, 665 m, steppe-rocky	April-May

Table 2. Pollen characteristics of *Trifolium* taxa

	Taxa	Polar axis (P) (µm)			Equatorial axis (E) (µm)			P / E ratio and Pollen shape	Aperture type	Ornamentation		Colpus		Porus		Exine	Intine
		Min.	Mean	Max.	Min.	Mean	Max.			Polar area and Apertur surroundings	Equatorial area	Clg	Clt	Plg	Plt		
1	*T. boissieri*	25.41	29.19	34.75	20.83	23.45	25.25	1.26/ subprolate	Trizonocolporate	Perforate	Microreticulate	17.41	2.5	6.0	4.83	0.77	0.35
2	*T. dasyurum*	26.91	38.67	48.91	21.41	33.79	37.0	1.07/ prolate-Spheroidal	Trizonocolporate	Reticulate	Reticulate	28.41	4.5	12.41	11.58	0.87	0.53
3	*T.scabium*	35.61	38.45	41.14	28.38	29.10	30.28	1.32/ subprolate	Trizonocolporate	Perforat. Psilate-Perforate	Microreticulate	25.09	2.48	5.99	7.47	0.78	0.43
4	*T. spumosum*	25.77	33.25	38.19	25.42	27.08	29.42	1.22/ subprolate	Trizonocolporate	Perforate. Psilate-Perforate	Reticulate	26.43	3.10	8.28	7.53	0.72	0.41
5	*T.pauciflorum*	32.44	33.84	34.55	28.66	29.74	30.88	1.13/ prolate-Spheroidal	Trizonocolporate	Perforate	Microreticulate	25.8	3.22	11.75	9.52	0.53	0.28

* Clg: Colpus width, Plg: Pore width, * Clt: Colpus length, Plt: Pore length

It is seen that the examined *Trifolium* taxa have significant similarities and differences when their pollen morphologies are mixed. Pollen shape, aperture structure, and ornamentation types are significantly different among the taxa. Other studies examining the pollen morphology of the genus *Trifolium* also show that these characters have important taxonomic diversity.

Koçyiğit et al. (2013) studied the pollen morphology of 16 *Trifolium* taxa collected from Istanbul province. Pollen shapes in the examined taxa were determined as prolate, subprolate, and spheroidal. Ornamentation was determined as reticulate and scabrate. Polar axis lengths were determined between 37.83 µm- 20.41 µm, while equatorial axis lengths were determined between 41.25 µm- 22.10 µm.

Gazar (2003) grouped *Trifolium* taxa collected in Egypt into two subclasses according to pollen morphology. The pollen of the taxa in the first group was circular and triangular in polar view and had a trizonocolporate aperture structure, while the pollen of the second group was semicircular-circular and semitriangular in polar view and had a tricolpate aperture structure.

Another study conducted in Egypt examined the pollen morphology of 12 *Trifolium* taxa. Similarly, it was determined that the pollen had prolate-spheroidal, subprolate, and perprolate shapes. Aperture types were determined to be in different structures as tricolporate, tricolpate, and polycolpate (Taia, 2004).

4. Discussion and Conclusions

In this study, the palynologically compared different aspects of *T. scabrum, T.boissieri, T. dasyurum, T. pauciflorum*, and *T. spumosum* species were determined and tried to be clarified. Significant differences were determined among the pollen types of the examined taxa in terms of size, shape, ornamentation, and aperture measurements. However, all of the examined pollen grains of *Trifolium* taxa were determined to be radially symmetric, isopolar, and trizonocolporate

As a result of the study, similarities and differences of the species that are difficult to distinguish from each other morphologically were revealed by examining their palynological features. Systematics using only morphological characters can create many taxonomic difficulties. Palynological analyses can be a source for systematic studies to overcome these difficulties.

In this study, pollen morphologies of taxa were examined by light microscopy and scanning electron microscopy (SEM). In the literature review, no comprehensive studies were found to determine the pollen morphologies of *T. scabrum, T.boissieri, T. dasyurum, T. pauciflorum,* and *T. spumosum* species. For this reason, this study will guide and enlighten future studies.

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Conflict of interest: The authors declare that there is no conflict of interest.

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Research Article/Araştırma Makalesi

Investigating the Pharmacological Potential of *Micromeria myrtifolia* Boiss. & Hohen.: Phenolic Profiling and Biological Activity Assessments

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Abstract: *Micromeria myrtifolia* Boiss. & Hohen. is a valuable medicinal plant in Türkiye, recognized for its extensive applications across the country. In this study, plant samples were collected from Muğla, Türkiye and extracts from both the aerial parts and roots were prepared using ethanol. To elucidate their phytochemical composition, a comprehensive LC-MS/MS analysis was conducted. The results revealed that both extracts were quite rich in phenolic compounds. Notably, nicotiflorin was the major constituent in both extracts (12878.31±355.44 µg/g extract in the aerial parts and 47512.4±1311.34 µg/g extract in the roots) along with significant phenolic acids such as rosmarinic acid and caffeic acid as well as flavonoids like hesperidin. Moreover, both extracts demonstrated substantial antioxidant activities compared to the synthetic antioxidant compounds as evaluated by DPPH free radical scavenging, ABTS cation radical scavenging, and CUPRAC activity assays. Both aerial parts and root extracts also exhibited meaningful anti-glucosidase activity with 73.03±0.16% and 47.06±0.41%, respectively, at 2 mg/mL concentration. The root extract also showed moderate butyrylcholinesterase inhibitory activity. This study contributes to the existing body of knowledge with valuable insights regarding the phytochemical profile and biological activities of *M. myrtifolia*, paving the way for future research aimed at exploring its medicinal properties and potential uses in traditional and modern medicine.

Keywords: *Micromeria* Benth., LC-MS/MS, antioxidant, enzyme inhibition.

Micromeria myrtifolia Boiss. & Hohen. Bitkisinin Farmakolojik Potansiyelinin Araştırılması: Fenolik İçeriğinin ve Biyolojik Aktivitelerinin Belirlenmesi

Öz: *Micromeria myrtifolia* Boiss. & Hohen., Türkiye'nin önemli tıbbi bitkilerindendir ve ülke genelinde farklı tıbbi amaçlarla oldukça yaygın olarak kullanılmaktadır. Bu çalışmada, *M. myrtifolia* örnekleri Muğla, Türkiye'den toplanmış hem topraküstü kısımlarından hem de köklerden ayrı ayrı etanol ekstreleri hazırlanmıştır. Ekstrelerin fitokimyasal içeriklerini belirlemek için detaylı bir LC-MS/MS analizi yapılmıştır. Analiz sonuçlarına göre her iki ekstrenin de fenolik bileşikler açısından oldukça zengin olduğu belirlenmiştir. Her iki ekstrede de ana bileşen olarak nikotiflorin öne çıkmış ve topraküstü kısımlarından hazırlanan ekstrede 12878.31±355.44 µg/g, kök ekstresinde ise 47512.4±1311.34 µg/g nikotiflorin tespit edilmiştir. Ayrıca ekstrelerde rozmarinik asit ve kafeik asit gibi önemli fenolik asitler ile hesperidin gibi flavonoitlerin varlığı da belirlenmiştir. Ekstrelerin antioksidan aktiviteleri DPPH serbest radikal giderici, ABTS katyon radikali giderici ve CUPRAC aktivitesi metotları ile değerlendirilmiş, buna göre her iki ekstre de sentetik antioksidan bileşiklere kıyasla anlamlı antioksidan aktiviteler sergilemiştir. Bunun yanı sıra ekstreler enzim inhibisyon aktiviteleri açısından değerlendirildiğinde, ekstrelerin 2 mg/mL konsantrasyonda %73.03±0.16 (topraküstü) ve %47.06±0.41 (kök) oranında anti-glukozidaz aktivite gösterdiği tespit edilmiştir. Kök ekstresi ayrıca orta düzeyde butirilkolinesteraz inhibe edici aktivite de göstermiştir. Bu çalışma, *M. myrtifolia* bitkisinin kimyasal içeriği ve biyolojik aktiviteleri konusunda mevcut literatüre önemli katkılarda bulunmakla birlikte, bitkinin geleneksel ve modern tıp uygulamalarındaki yeni ve potansiyel kullanım alanlarını keşfetmeye yönelik gelecek araştırmalara da zemin hazırlamaktadır.

Anahtar kelimeler: *Micromeria* Benth., LC-MS/MS, antioksidan, enzim inhibisyonu.

1. Introduction

The Anatolian peninsula hosts the richest flora among the Middle Eastern countries. Current estimates place the number of taxa in Anatolia and Thrace (the European part of Türkiye) at around 11.750—about 2.000 more than those recorded by Davis in the Flora of Turkey (Yesilada, 2005). Alongside its diverse flora, the significant cultural diversity of Anatolia, which is shaped by its great historical events, forms the foundation of a vibrant tradition of medicinal practices in the region (Yeşilada, 2002).

Micromeria Benth. genus (Lamiaceae) is widely

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distributed, ranging from South Africa to Western Europe and Asia, and includes a large variety of perennial plants, with 70–90 species of dwarf shrubs and subshrubs. *Micromeria* species frequently grow wild in mountainous, open habitats or rocky areas around the world. In Europe, about twenty-two *Micromeria* species are present, with a significant concentration in the Balkan Peninsula (Mohammadhosseini et al., 2022). In Türkiye, *the Micromeria* genus is represented by nine species and fourteen taxa in Türkiye, nine of which are endemic (Duman & Dirmenci, 2017). Various *Micromeria* species have been reported to be utilized in the traditional medicinal practices of Anatolia for different purposes. For instance, *M. cristata* subsp. *orientalis* decoctions are used to treat ailments such as bronchitis, common colds, diabetes, headache, stomachache, kidney diseases, and prostrate disorders in the Eastern Blacksea Region. Infusions prepared with the aerial parts of *M. juliana* are also used to treat stomachache (Selvi et al., 2022). *M. fruticosa* aerial parts infusions are commonly applied as carminative and against nausea (Salim and Necattin, 2018). *M. graeca* subsp. *graeca* is used in veterinary medicine for skin problems, particularly wounds (Güzel et al., 2015).

Among the *Micromeria* species esteemed for their medicinal properties in Türkiye, *M. myrtifolia* Boiss. Et Hohen is considered as one of the most significant due to its diverse uses in traditional medicine. It possesses numerous local names, including "*kertiş kuyruğu, boğumlu çay, dağ çayı, kırkboğum, yeşil çay*, *Nurettin çayı*, *güvercin otu*, and *topuklu çay*" highlighting its status as a highly valued and widely utilized medicinal plant in Türkiye. Aerial parts of the plant have been used as an infusion for appetitive and carminative purposes. Leaf powder of the plant is applied to treat gallstones and gastrointestinal disorders. Besides, infusions are commonly consumed as medicinal tea for relaxation and pleasure. Infusions are also thought to be beneficial against cold, flu, and sore throat. Flowers and leaves of the plant have been recommended for shortness of breath (Polat & Satil, 2012; Güzel et al., 2015; Sargin, 2015; Kocabaş & Gedik, 2016; Salim & Necattin, 2018; Sargin & Büyükcengiz, 2019; Selvi et al., 2022; Baykan et al., 2023).

Belonging to the Lamiaceae family, *Micromeria* species are renowned for being aromatic plants. Therefore, they are noted for being a rich source of essential oils and the research has been mainly focused on their essential oil and their components. Monoterpenes, sesquiterpenes, and their oxygenated derivatives have been determined as major constituents. The concentrations of these compounds differ based on the subspecies. Along with the volatile secondary metabolites, *Micromeria* species are also characterized by containing high amounts of phenolic compounds. Chlorogenic acid and rosmarinic acid are among the most commonly found phenolic acids. Hesperetin, naringin, and quercetin are the mainly determined flavonoids in different *Micromeria* species (Hamwi & El-Lakany, 2021). Diverse phytochemicals found in *Micromeria* species are responsible for their important biological activities. To this point, different extracts prepared with *Micromeria* species have been shown to exhibit antioxidant, antimicrobial, anti-inflammatory, cytotoxic, analgesic, insecticidal, and antidepressant activities (Küpeli Akkol et al., 2019, Yilmaz et al., 2024). There have also been studies that concentrate specifically on *M. myrtifolia* in this regard. Accordingly, *M. myrtifolia* possesses antioxidant, antidepressant, enzyme inhibitory, antifungal, cytogenetic, and cytotoxic properties (Özcan, 1999; Formisano et al., 2014; Küpeli Akkol et al., 2019; Sarikurkcu et al., 2020a).

The current study begins with the preparation of ethanol extracts from both the aerial parts and roots of *M. myrtifolia* collected from Köyceğiz-Muğla/Türkiye. We aim to provide a comprehensive analysis of the chemical constituents through liquid chromatography-mass spectrometry, while also investigating the total phenolic and flavonoid content, as well as the diverse biological activities of the studied extracts, including antioxidant, anticholinesterase, antityrosinase, antiurease, and anti-glucosidase properties. By elucidating the multifaceted benefits of these extracts, we hope to highlight the potential of *M. myrtifolia* as a valuable source of natural bioactive compounds, thereby contributing to the growing body of knowledge surrounding its therapeutic applications and traditional medicinal uses in Türkiye.

2. Material and Method

2.1. Plant Material

Micromeria myrtifolia Boiss. & Hohen. aerial parts and roots were collected during the flowering phase by Assoc. Prof. Yeter Yeşil (Istanbul University, Faculty of Pharmacy, Pharmaceutical Botany Department) and Prof. Dr. Mehmet Boğa (Dicle University, Faculty of Pharmacy, Analytical Chemistry Department) in June 2014, approximately 100 meters past the Namnam Bridge, on the left side of the Köyceğiz-Muğla road. The plant was identified by Assoc. Prof. Yeter Yeşil and samples have been preserved in the Herbarium of Istanbul University Faculty of Pharmacy (ISTE No: 116045).

2.2. Preparation of the Extracts

The aerial and root parts of *M. myrtifolia* were shade-dried and subsequently pulverized. Dry weights were determined to be 22.20 g for the aerial parts and 32.30 g for the root parts. The powdered samples were then subjected to maceration with ethanol (96%) at room temperature for three consecutive 24-hour periods. Solvent removal was performed using a rotary evaporator (Heidolph, Germany), yielding the extracts in dry form. The extraction yield (%) was calculated according to the following equation:

Yield (%) (w/w) = (Amount of extract / Amount of dry plant material) x 100

2.3. Chemicals and Instruments

The chemical composition of the extracts was analyzed via LC-MS/MS (Shimadzu, Kyoto, Japan). *In vitro* biological activity assays were performed using a Shimadzu UV spectrophotometer and a BioTek PowerWave XS microplate reader (USA). Compounds for LC-MS/MS analysis and biological assays were obtained from Merck (Germany), Sigma (Germany), and Fluka (Germany). All solvents were of analytical grade.

2.4. LC-MS/MS Analysis

The LC-MS/MS analysis of the extracts was conducted by a previously validated protocol (Yilmaz et al., 2018). Quantification of 37 phenolic constituents was achieved

via a Nexera UHPLC system (Shimadzu) coupled with a tandem mass spectrometer. Data acquisition and processing were performed using Shimadzu Lab Solutions software, leveraging the capabilities of the LC-ESI-MS/MS platform for detailed compound profiling.

2.5. Total Phenolic and Flavonoid Contents of the Extracts

The total phenolic content of the extracts was determined as micrograms of pyrocatechol equivalents (PEs) using the method developed by Slinkard and Singleton (1977). Details: Volumes of 0, 1, 2, 3, 4, 5, 6, 7, and 8 µL of 100 ppm pyrocatechol were adjusted with water to achieve a final volume of 184 µL. Following this, 4 µL of Folin-Ciocalteu reagent and 12 µL of 2% Na_2CO_3 were added to each mixture. After a 2-hour incubation period, absorbance was measured at 760 nm. Total flavonoid contents were expressed as micrograms of quercetin equivalents (QEs), calculated by the method described by Moreno et al. (2000). Details: Volumes of 0, 1, 2, 3, 4, 5, 6, 7, and 8 µL of 1000 ppm quercetin were combined with 80% ethanol to bring the total volume to 192 µL. Following this, 4 µL of 1 M potassium acetate was added, and after 1 minute, 4 µL of 10% aluminium nitrate was incorporated. After a 40-minute incubation period, absorbance readings were taken at 415 nm.

To calculate the total phenolic and flavonoid contents of the extract, the equations below were used:

Absorbance = 0.0409 pyrocatechol (µg) + 0.0495 (R^2 = 0.9975)

Absorbance = 0.0347 quercetin (µg) + 0.1174 (R^2 = 0.9951)

The calibration curves for pyrocatechol and quercetin to calculate the total phenolic and flavonoid contents are given in Fig. 1 and Fig. 2.

2.6. Antioxidant Activity Assays

The antioxidant activity of the ethanol extracts from the aerial and root parts of *M. myrtifolia* was assessed using the DPPH free radical scavenging, ABTS cation radical scavenging, and CUPRAC activity methods. Absorbance measurements were performed with a UV-Vis spectrophotometer (PG Instruments T80+ UV/VIS Spectrometer, UK) and an ELISA reader (BioTek EON Microplate Reader, USA).

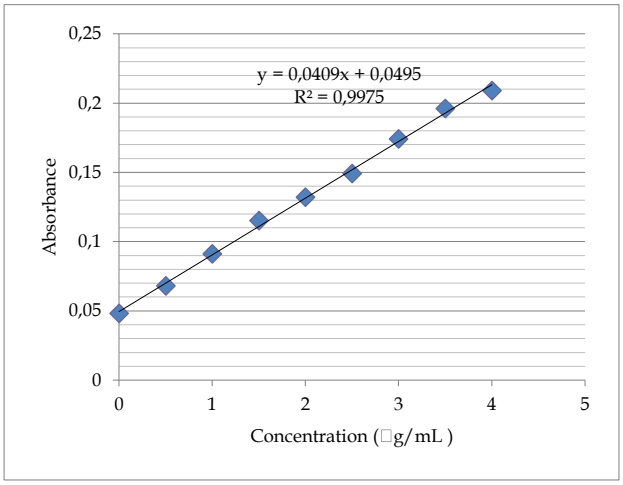

Figure 1. Pyrocatechol Calibration Curve for Total Phenolic Content

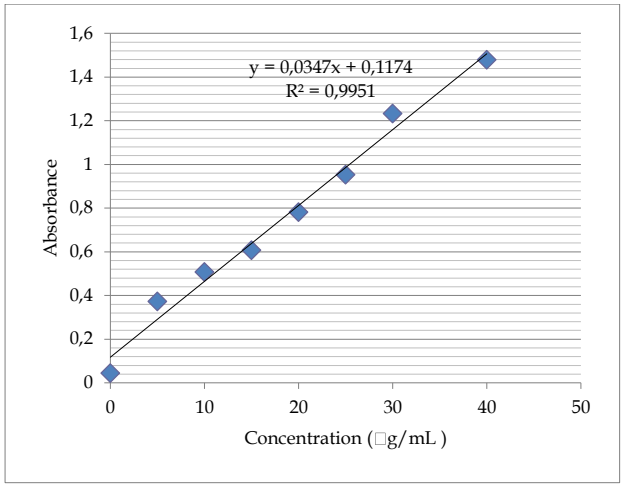

Figure 2. Quercetin Calibration Curve for Total Flavonoid Content

2.6.1. DPPH Free Radical Scavenging Activity

The free radical scavenging activity of the aerial and root parts of *M. myrtifolia* against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was evaluated using the method designed by Blois (1958) with modifications (Eroğlu Özkan et al., 2022). Stock solutions of the extracts were prepared at a concentration of 1000 µg/mL. Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and α-tocopherol served as standards. Details: Volumes of 2, 5, 10, and 20 µL of the stock solution were prepared. The volumes were adjusted to 40 µL using ethanol, then 160 µL of 0.1 mM DPPH was added. After a 30-minute incubation at room temperature in the dark, the absorbance was measured at 517 nm.

The following equation was used to calculate the DPPH free radical scavenging potential:

DPPH scavenging effect (Inhibition %) = $(A_{control} - A_{sample})/A_{control}$ x 100

$A_{control}$ = Absorbance of the control

A_{sample} = Absorbance of the samples

2.6.2. ABTS Cation Radical Scavenging Activity

The cation radical scavenging activity of the aerial and root parts of *M. myrtifolia* was assessed using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by applying the method designed by Re et al. (1999) with modifications (Ersoy et al., 2022). Stock solutions of the extracts were prepared at a concentration of 1000 µg/mL. BHT, BHA, and α-Toc were used as standards. Details: Volumes of 2, 5, 10, and 20 µL of the stock solution were prepared in triplicate. The total volume was adjusted to 40 µL with ethanol, then 160 µL of 7 mM ABTS was added. After a 6-minute incubation in the dark, the change in absorbance was measured at 734 nm.

The following equation was used to calculate the ABTS cation radical scavenging potential:

ABTS cation radical scavenging effect (Inhibition %) = $(A_{control} - A_{sample})/A_{control}$ x 100

$A_{control}$ = Absorbance of the control

A_{sample} = Absorbance of the samples

2.6.3. CUPRAC Activity

The CUPRAC activity of the aerial and root parts of *M. myrtifolia* was assessed using the method developed by Apak et al. (2004). BHT, BHA, and α-Toc were used as standards. Details: Volumes of 2.5 µL, 6.25 µL, 12.5 µL, and 25 µL of the stock solution were prepared. The total volumes were adjusted to 67 µL with distilled water, followed by the addition of 61 µL of 0.01 M $Cu(II)Cl_2$, 61 µL of 0.1 M NH_4CH_3COO, and 61 µL of 0.1 M neocuproine. After a 1-hour incubation, the change in absorbance was measured at 450 nm.

2.7. Enzyme Inhibitory Activity Assays

The following equation was used to calculate the enzyme inhibition % in all tests:

$(A_{control} - A_{sample})/A_{control} \times 100$

$A_{control}$ = Absorbance of the control

A_{sample} = Absorbance of the samples

2.7.1. Acetylcholinesterase and Butyrylcholinesterase Inhibitory Activities

For the assessment of acetylcholinesterase and butyrylcholinesterase inhibitory activities of the aerial and root parts of *M. myrtifolia*, a rapid colorimetric method known as Ellman (Ellman et al., 1961) with modifications (Ersoy et al., 2020) was employed. This method is based on the measurement of absorbance of the yellow-colored 5-thio-2-nitrobenzoate anion formed by the reaction of thiocholine released from acetylcholine hydrolysis with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). After preparing stock solutions of the extracts at 4000 µg/mL, experiments were conducted three times. Galantamine was used as the standard. Details: The assay procedure began by adding 150 µL of 100 mM sodium phosphate buffer (pH 8) to a reaction vessel, followed by the addition of 10 µL of the sample (4000 µg/mL) or control. Next, 20 µL of the enzymes were added and the mixture was incubated at 25°C for 15 minutes. Following this initial incubation, 10 µL of 5 mM DTNB was introduced, along with 10 µL of 7.1 mM acetylthiocholine iodide (0.79 mM butyrylthiocholine iodide). The mixture was then incubated at 25°C for another 10 minutes before the absorbance was measured at 412 nm.

2.7.2. Tyrosinase Inhibitory Activity

To evaluate tyrosinase inhibition activity, tyrosinase enzyme was utilized as the enzyme and L-DOPA acted as the substrate. In the microplate wells, 10 µL of the sample, 150 µL of phosphate buffer (pH 6.8) and 25 µL of the enzyme solution prepared in the buffer were combined. This mixture was stirred for 3 minutes and then incubated at 37°C for 10 minutes. Following this incubation, 20 µL of L-DOPA was added as the substrate. After an additional 10 minutes at 37°C, the change in absorbance was measured at 475 nm. Kojic acid was used as the standard. This method was first reported by Masamoto and Kubo (1980).

2.7.3. Urease Inhibitory Activity

To measure urease inhibition activity, urease enzyme was used along with urea as the substrate. In the microplate wells, 10 µL of the sample, 25 µL of the enzyme mixed in phosphate buffer (pH = 8) and 50 µL of urea were added together. The mixture was then incubated at 30°C for 15 minutes. Afterward, 45 µL of phenol reagent and 70 µL of alkaline reagent were added. After another 20 minutes, the microplate was placed in an ELISA reader and the absorbance was measured at 630 nm. The details of this procedure were given by Weatherburn (1967).

2.7.4. α-Glucosidase Inhibitory Activity

The α-glucosidase inhibition activity was assessed following the method described by Schmidt et al. (2012). Details: In each well, 90 µL of 0.1 M phosphate buffer (pH 7.5; 0.02% NaN_3), 10 µL of the sample and 80 µL of the enzyme solution (with a final concentration of 0.05 U/mL in the well) were added. The mixture was incubated at 28°C for 10 minutes. Subsequently, 20 µL of PNPG (with a final concentration of 1.0 mM in the well) was added, and the mixture was incubated for an additional 35 minutes. Absorbance was measured at 405 nm, with acarbose used as the standard.

2.8. Statistical Analysis

The biological activity results are presented as the mean and standard deviation of three parallel measurements. The results were evaluated within a 95% confidence interval according to the Student t-test, with a significance threshold of $p < 0.05$. Linear regression analysis, using the least squares method, was performed to assess the slope, intercept, and correlation coefficients.

3. Results and Discussion

The LC-MS/MS analysis of ethanol extracts from the aerial parts and roots of *M. myrtifolia* was conducted following the method optimized and validated by Yilmaz et al. (2018). Table 1 presents the analytical parameters and results of these analyses and Figs. 3, 4, and 5 show the chromatograms for the standard compounds and studied extracts. Both extracts were found to be quite rich in terms of biologically active phytochemicals.

In *M. myrtifolia* aerial parts extract, 18 different secondary metabolites were determined. The most abundant constituent was revealed as nicotiflorin with 12878.31±355.44 µg/g extract. Other major components were found to be quinic acid (8171.29±67.00 µg/g extract), rosmarinic acid (6620.59±472.05 µg/g extract), naringenin (2532.19±131.93 µg/g extract), malic acid (917.44±10.37 µg/g extract). Moreover, hesperidin (570.28±14.94 µg/g extract), caffeic acid (580.79±20.56 µg/g extract), fumaric acid (564.63±7.00 µg/g extract), rhoifolin (544.73±51.26 µg/g extract), apigenin (235.19±15.29 µg/g extract), protocatechuic acid (195.83±8.05 µg/g extract), vanillin (80.08±2.24 µg/g extract), isoquercitrin (69.07±0.92 µg/g extract), salicylic acid (35.44±1.17 µg/g extract), fisetin (18.2±0.27 µg/g extract), hesperetin (17.75±1.00 µg/g extract), quercetin (8.19±0.44 µg/g extract), and *p*-coumaric acid (0.65±0.03 µg/g extract).

In *M. myrtifolia* root extract, 16 different components were identified. Similar to the aerial parts extract, nicotiflorin was the primary constituent with a concentration of 47512.4±1311.34 µg/g extract. Further major compounds were determined as quinic acid (1762.88±14.46 µg/g extract), rosmarinic acid (1527.81±108.93 µg/g extract),

rhoifolin (1067.04±100.41 µg/g extract), and hesperidin (726.54±19.04) µg/g extract). Malic acid (594.28±6.72 µg/g extract), fumaric acid (527.90±6.55 µg/g extract), caffeic acid (199.72±7.07 µg/g extract), vanillin (140.52±3.93 µg/g extract), protocatechuic acid (59.67±2.45 µg/g extract), *p*-coumaric acid (49.62±2.56 µg/g extract), apigenin (25.52±1.66 µg/g extract), hesperetin (23.24±1.31 µg/g extract), naringenin (15.36±0.80 µg/g extract), salicylic acid (12.93±0.43 µg/g extract), and isoquercitrin (8.63±0.11 µg/g extract).

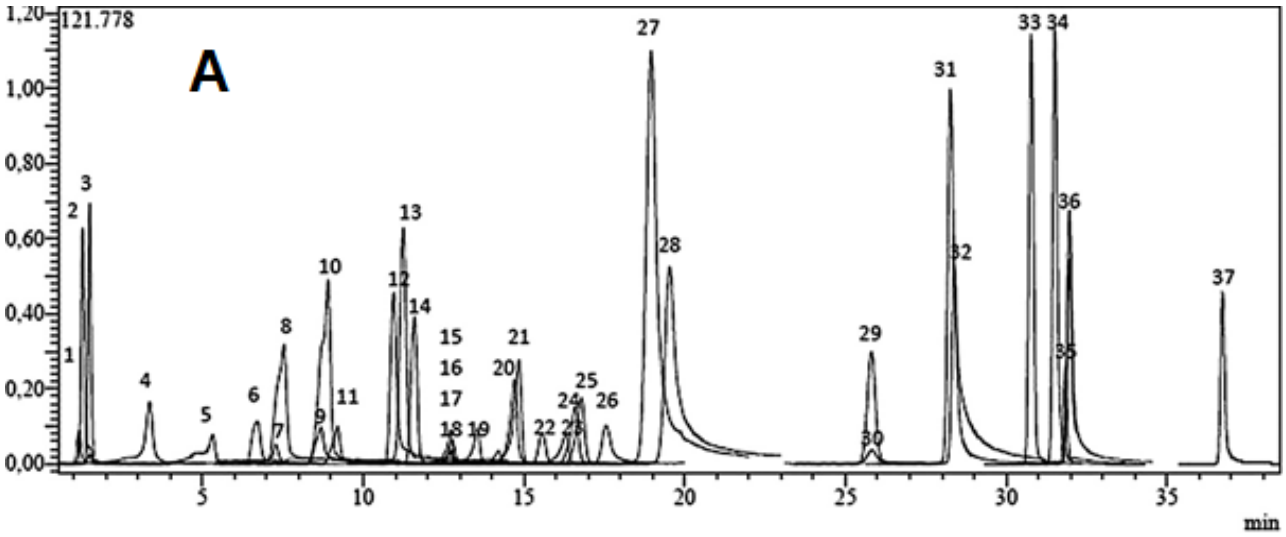

Figure 3. LC-MS/MS Chromatogram of the Standard Compounds

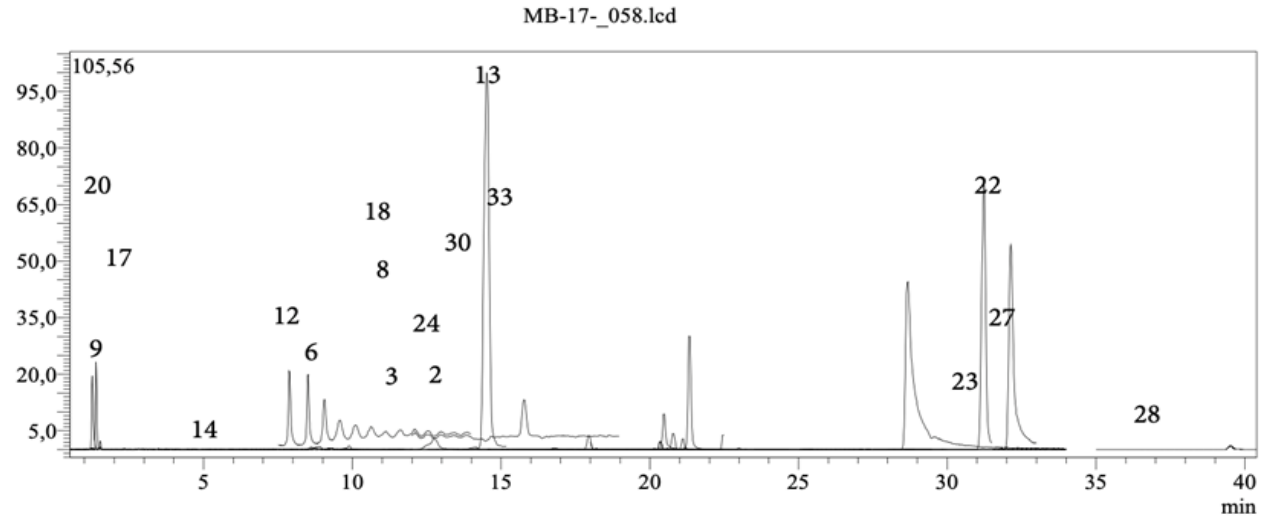

Figure 4. LC-MS/MS Chromatogram of *M. myrtifolia* Aerial Parts Extract

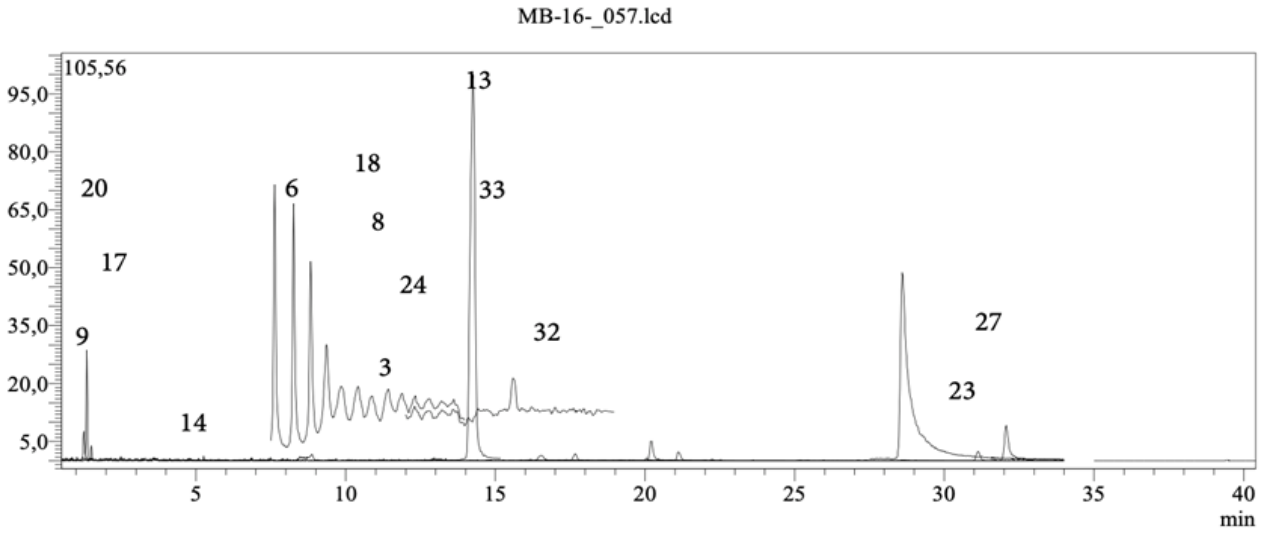

Figure 5. LC-MS/MS Chromatogram of *M. myrtifolia* Roots Extract

Table 1. LC-MS/MS Results of the Studied Extracts with Analytical Parameters

	Compounds	RT[a]	Parent ion (m/z)	Daughter Ions	Ion. Mode	Quantification(µg analyte/g extract)[b]	
						M. myrtifolia aerial parts	*M. myrtifolia* roots
1	**Coumarin**	17.40	147.05	91.0-103.2	Pos	ND	ND
2	**Hesperidin**	12.67	610.90	303.1-465.1	Pos	570.28±14.94	726.54±19.04
3	**p-coumaric acid**	11.53	162.95	119.25-93.25	Neg	0.65±0.03	49.62±2.56
4	**o-coumaric acid**	15.45	162.95	119.35-93.25	Neg	ND	ND
5	**Gallic acid**	3.00	168.85	125.2-79.2	Neg	ND	ND
6	**Caffeic acid**	8.80	178.95	135.2-134.3	Neg	580.79±20.56	199.72±7.07
7	**Vanillic acid**	8.57	166.90	152.25-108.25	Neg	ND	ND
8	**Salicylic acid**	11.16	136.95	93.3-65.3	Neg	35.44±1.17	12.93±0.43
9	**Quinic acid**	1.13	190.95	85.3-93.3	Neg	8171.29±67.00	1762.88±14.46
10	**4-OH-Benzoic acid**	7.39	136.95	93.3-65.3	Neg	ND	ND
11	**tr-Ferulic acid**	12.62	192.95	178.3	Neg	ND	ND
12	**Chlorogenic acid**	7.13	353.15	191.2	Neg	ND	ND
13	**Rosmarinic acid**	14.54	359.00	161.2-197.2	Neg	6620.59±472.05	1527.81±108.93
14	**Protocatechuic acid**	4.93	152.95	108.3	Neg	195.83±8.05	59.67±2.45
15	**Cinnamic acid**	25.61	147.00	103.15-77.3	Neg	ND	ND
16	**Sinapinic acid**	12.66	222.95	208.3-149.2	Neg	ND	ND
17	**Fumaric acid**	1.48	115.00	71.4	Neg	564.63±7.00	527.90±6.55
18	**Vanillin**	10.87	151.00	136.3-92.2	Neg	80.08±2.24	140.52±3.93
19	**Pyrocatechol**	6.48	109.00	108.35-91.25	Neg	ND	ND
20	**Malic acid**	1.23	133.00	115.2-71.3	Neg	917.44±10.37	594.28±6.72
21	**Syringic acid**	9.02	196.95	182.2-167.3	Neg	ND	ND
22	**Hesperetin**	31.76	300.95	164.2-136.2	Neg	17.75±1.00	23.24±1.31
23	**Naringenin**	30.68	270.95	151.2-119.3	Neg	2532.19±131.93	15.36±0.80
24	**Rutin**	12.61	609.05	300.1-271.1	Neg	ND	ND
25	**Quercetin**	28.17	300.90	151.2-179.2	Neg	8.19±0.44	ND
26	**Quercitrin**	16.41	447.15	301.15-255.15	Neg	ND	ND
27	**Apigenin**	31.43	268.95	117.3-151.2	Neg	235.19±15.29	25.52±1.66
28	**Chrysin**	36.65	252.95	143.3-119.4	Neg	ND	ND
29	**Liquiritigenin**	25.62	254.95	119.25-135.15	Neg	ND	ND
30	**Isoquercitrin**	13.42	463.00	300.15-271.15	Neg	69.07±0.92	8.63±0.11
31	**Cosmosiin**	16.59	431.00	268.2-239.2	Neg	ND	ND
32	**Rhoifolin**	16.11	577.05	269.2-211.15	Neg	544.73±51.26	1067.04±100.41
33	**Nicotiflorin**	14.68	593.05	285.1-255.2	Neg	12878.31±355.44	47512.4±1311.34
34	**Fisetin**	19.30	284.95	135.2-121.25	Neg	18.2±0.27	ND
35	**Luteolin**	28.27	284.75	133.2-151.2	Neg	ND	ND
36	**Myricetin**	18.72	317.00	179.15-151.25	Neg	ND	ND
37	**Kaempferol**	31.88	284.75	255.1-117.3	Neg	ND	ND

[a]RT: Retention time, [b]Values in µg/g (w/w) of plant extracts.

Sarikurkcu et al. (2020a) carried out an LC–ESI–MS/MS analysis to investigate the phenolic constituents of *M. micromaria* aerial parts (from Muğla-Türkiye) water, methanol, and ethyl acetate extracts. Accordingly, the methanol and water extracts were particularly rich in rosmarinic, syringic, chlorogenic, caffeic, and protocatechuic acids, whereas rosmarinic acid and apigenin were the predominant compounds in the ethyl acetate extract. Küpeli Akkol et al. (2019) also conducted a study aiming to isolate the secondary metabolites of *M. myrtifolia* aerial parts collected from Antalya-Türkiye. Three extracts (methanol, ethyl acetate, and n-hexane) were prepared. Reportedly, rosmarinic acid, naringenin, apigenin, and myricetin were isolated from the methanol extract. In another study, *M. myrtifolia* methanol extract was shown to contain high amounts of rosmarinic acid, quercetin, and chlorogenic acid (Taskin et al., 2024). It can be said that the findings of the current study demonstrate a high degree of concordance with the results reported in previous studies.

Other *Micromaria* species were also investigated for their phenolic constituents. Speaking of which, gallic acid, caffeic acid, chlorogenic acid, rosmarinic acid, diosmin, and apigenin were identified in *M. graeca* aerial parts from Algeria (ethanol extract) by an HPLC analysis (Brahmi et al., 2017). In another study, different extracts (methanol, water, and ethyl acetate) of *M. nervosa* from Türkiye were shown to be rich in terms of rosmarinic acid. Besides, luteolin, apigenin, chlorogenic acid, protocatechuic acid, caffeic acid, and vanillic acid were determined in the extracts (Sarikurkcu et al., 2020b). In *M. inodora* extract, different phenolic acids and flavonoids, predominantly gallic acid, quercetin, rutin, vanillin, and naringenin were detected by an RP-HPLC-PDA analysis (Adjdir et al., 2021). In *M. fruticosa* (from Palestine) methanol extract, phenolic compounds including gallic acid, chlorogenic acid, catechin, protocatechuic acid, rosmarinic acid, apigenin, and quercetin were screened (Abu-Gharbieh & Ahmed, 2016). In a recent study by Yilmaz et al. (2024), an ethanol extract of *M. cymuligera* (from Türkiye, endemic) was found to contain notable amounts of rosmarinic acid, quinic acid, chlorogenic acid, and cynaroside. Incidentally, these species were thought to be extinct for almost 150 years until samples were rediscovered in 2011. In *M. frivaldszkyana* (from Bulgaria, endemic) aerial parts ethanol extract, rosmarinic acid was revealed as the major constituent. Furthermore, vanillic acid, caffeic acid, chlorogenic acid, protocatechuic acid, salicylic acid, hesperidin, (-)-epicatechin, and apigenin were reported to be present in the extract (Mladenova et al., 2021).

Focusing on the current study, the abundance of nicotiflorin in both extracts is remarkable, highlighting its contribution to the bioactivity of the extracts. Nicotiflorin (kaempferol-3-β-rutinoside) is a flavonoid glycoside with well-established anti-inflammatory, antioxidant, antibacterial, antiviral, analgesic, anti-hypertensive, anti-anaphylactic, and neuroprotective effects. Medicinal plants that are rich in nicotiflorin have been used for the treatment and prevention of cardiovascular diseases in Traditional Chinese Medicine (Li et al., 2006; Yu et al., 2021). Incidentally, to the authors' best knowledge, this is the first report indicating the significant presence of nicotiflorin in *Micromeria* species. Sarikurkcu et al. (2020a) did not report the determination of nicotiflorin in *M. myrtifolia* in their analysis. Yilmaz et al. (2024) did not detect any nicotiflorin in *M. cymuligera* extract although it was among the standard compounds.

The composition, content, and proportions of bioactive compounds within a single plant species may vary significantly due to the influence of diverse environmental factors in their habitats. Additionally, a notable correlation exists between the flavonoid profiles of plants and their ecological and morphological attributes (Mykhailenko et al., 2020). As a case in point, Ersoy et al. (2023) indicated significant differences in the phenolic compositions of *Teucrium multicaule* samples collected from two different locations in Türkiye. Nevertheless, while present phenolic compounds can vary due to numerous factors, the compositions of *Micromeria* species were observed to be notably similar to one another. Brahmi et al. (2017) also emphasized this situation. In their study, *Micromeria* species in general were reported to contain mostly phenolic acids (such as gallic, chlorogenic, vanillic, ferulic, *and p*-coumaric acids) and flavonoids (such as apigenin, luteolin, naringenin, hesperetin, and quercetin). As additional studies investigating the phenolic constituents of different *Micromeria* species emerge, a clearer understanding will be developed on this subject.

The results of the calculation of total phenolic and flavonoid contents of *M. myrtifolia* aerial parts and root extracts as well as the extract yields were given in Table 2. The ethanol extract of *M. myrtifolia* aerial parts exhibited a total phenolic content of 58.07±1.46 µg PEs/mg extract, while the total flavonoid content for the same extract was determined to be 16.17±0.83 µg QEs/mg extract. In contrast, the ethanol extract of *M. myrtifolia* roots showed a total phenolic content of 50.12±1.59 µg PEs/mg extract, with a total flavonoid content calculated at 25.10±1.02 µg QEs/mg extract. Both extracts were found to contain high amounts of total phenolics and flavonoids.

Table 2. Yields (%), Total Phenolic and Flavonoid Contents of the Studied Extracts

Extracts	Yield %	Phenolic content (*µ*g PEs/mg extract)[a]	Flavonoid content (*µ*g QEs/mg extract)[b]
M. myrtifolia aerial parts	3.69	58.07±1.46	16.17±0.83
M. myrtifolia roots	0.98	50.12±1.59	25.10±1.02

*Values expressed are means ± standard deviation of three parallel measurements (p<0.05)

[a] PEs, pyrocatechol equivalents (y = 0.0409x + 0.0495, R^2 = 0.9975).

[b] QEs, quercetin equivalents (y = 0.0347x + 0.1174, R^2 = 0.9951)

Studies in this area have revealed that different plant organs possess varying levels of total phenolic and flavonoid contents. For instance, aerial parts of *Hypericum empetrifolium* were found to be richer than the roots of the plant in terms of total phenolic and flavonoid contents (Boga et al., 2021). Total phenolic and flavonoid contents of *Thymus praceox* subsp. *grossheimi* aerial parts and roots and *T. pubescens* aerial parts and roots were significantly different than each other (Eroglu Ozkan et al., 2022). Likewise, fruits and leaves of *H. androsaemum* were shown to contain different amounts of total phenolic and flavonoids (Bektaş et al., 2021).

Regarding total phenolic and flavonoid contents of different *Micromeria* species, in a study carried out with *M. croatica, M. juliana* and *M. thymifolia* (from Croatia), the dried plant samples were analyzed spectrophotometrically to determine their total contents of polyphenols (9.69–13.66%), phenolic acids (5.26–6.84%), flavonoids (0.01–0.09%), and tannins (3.07–6.48%) (Vladimir-Knežević et al., 2011). Total phenolic contents across different extracts of *M. graeca* were found to vary between 211 ± 11.9 mg GAE/g and 360 ± 22.1 mg GAE/g. The water extract exhibited the highest, whereas the ethyl acetate extract displayed the lowest total phenolic content. These results also indicate the influence of solvent on the total phenolic and flavonoid contents of the extracts (El Kamari et al., 2024).

Antioxidant activity of the aerial parts and root extracts of *M. myrtifolia* was determined using three different methodologies: DPPH free radical scavenging, ABTS cation radical scavenging, and CUPRAC activity assays.

The results of the DPPH free radical scavenging activity are presented in Fig. 6, pointing out the antioxidant potential of the ethanol extracts from both the aerial parts and roots of *M. myrtifolia*. These findings demonstrate the ability of extracts to scavenge DPPH free radicals, which is crucial for understanding their potential therapeutic applications regarding antioxidant activity. The graphical representations of the results, including comparisons to the standards BHT, BHA, and α-TOC, can be found in Fig. 6, illustrating the comparative efficacy of each extract in scavenging DPPH radicals.

As shown in Fig. 6, the ethanol extracts from *M. myrtifolia* aerial parts and roots demonstrated varying levels of inhibition activity across concentrations. At 10 µg/mL, *M. myrtifolia* aerial parts and roots exhibited 19.99% and 31.37% inhibition, respectively; at 25 µg/mL, they showed 40.96% and 51.72%; at 50 µg/mL, 76.11% and 73.09%; and at 100 µg/mL, 87.61% and 85.81%. At lower concentrations (10 and 25 µg/mL), both extracts had lower activity than all three standard compounds (BHT, BHA, and α-TOC). However, at 50 µg/mL, the activity of *M. myrtifolia* roots extract approached that of BHT, while aerial parts extract surpassed BHT. At the highest concentration, 100 µg/mL, both extracts exhibited DPPH radical scavenging activities similar to BHA and α-TOC and higher than BHT. Overall, these results indicate that both *M. myrtifolia* aerial parts and roots—extracts demonstrate good antioxidant activity, highlighting their potential as natural sources of antioxidants.

ABTS cation radical scavenging activity of ethanol extracts from the aerial parts and roots of *M. myrtifolia* are presented in Fig. 7, highlighting their antioxidant potential. Fig. 7 illustrates the scavenging efficiency of each extract, compared to standards BHT, BHA, and α-TOC, underscoring their promising antioxidant properties.

As shown in Fig. 7, the ethanol extracts from *M. myrtifolia* aerial parts and roots exhibited ABTS cation radical scavenging activities of 65.64% and 59.22% at 10 µg/mL, 86.63% and 85.12% at 25 µg/mL, 88.59% and 87.08% at 50 µg/mL, and 88.77% and 88.06% at 100 µg/mL, respectively. At concentrations of 10 and 25 µg/mL, the *M. myrtifolia* aerial parts extract demonstrated higher activity than the standards BHT and TOC, while at 50 and 100 µg/mL, it showed activity comparable to TOC and greater than BHT. The *M. myrtifolia* roots extract, on the other hand, exhibited higher activity than BHT and was close to TOC at 10 µg/mL, surpassed both standards at 25 µg/mL, and maintained levels near the other standards at 50 and 100 µg/mL.

The results demonstrate that both the ethanol extracts from the aerial parts and roots of *M. myrtifolia* possess significant ABTS cation radical scavenging activity, indicating their potential as effective natural antioxidants. The dose-dependent increase in scavenging activity suggests that higher concentration enhances their antioxidant effects. Notably, *M. myrtifolia* aerial parts extract exceeded the standards of BHT and TOC at lower concentrations and maintained comparable activity at higher concentrations. Meanwhile, *M. myrtifolia* roots extract also showed considerable antioxidant potential, particularly at 25 µg/mL. These findings highlight *M. myrtifolia* as a promising candidate for further research and development in antioxidant applications, reinforcing its value in both traditional and modern medicine.

Fig. 8 presents the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) assay results, assessing the reducing power of ethanol extracts from the aerial parts and roots of *M. myrtifolia*. By measuring the ability of the extracts to reduce cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}), it is possible to gain insights into their antioxidant activity. The results, shown in Fig. 8, illustrate the antioxidant capacities of both extracts at different concentrations, allowing comparisons with established standards.

At a concentration of 10 mg/mL, the aerial parts (0.55) and roots (0.63) of *M. myrtifolia* demonstrated notable reducing power, significantly lower than that of the standards BHA (1.25) and BHT (1.98), while α-TOC had a value of 0.48. As the concentration increased to 25 mg/mL, the reducing power of both extracts improved, with values of 1.11 for aerial parts and 1.31 for roots, indicating that they retained their antioxidant capacity but were still lower than BHA (2.35) and BHT (3.68), while surpassing α-TOC (1.06). At 50 mg/mL, both extracts showed enhanced activity, reaching values of 2.11 for aerial parts and 2.46 for roots, still below the standards BHA (3.63) and BHT (3.99). Notably, at 100 mg/mL, both extracts exhibited significant antioxidant activity with values of 3.37 for aerial parts and 3.99 for roots, aligning with the performance of the BHT and BHA standards, which also reached 3.99. These findings suggest that *M. myrtifolia* extracts possess promising antioxidant activity, particularly in the root extract, which demonstrates significant reducing power, especially at higher concentrations.

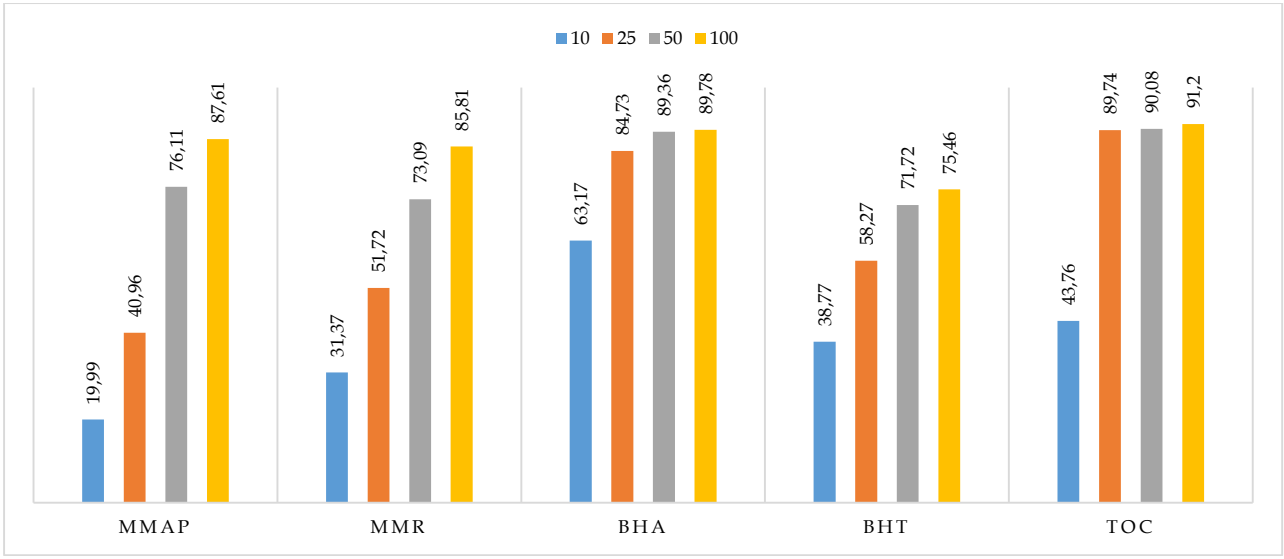

Figure 6. DPPH Free Radical Scavening Activity Results (Concentration-Inhibition%), MMAP: *M. myrtifolia* Aerial Parts, MMR: *M. myrtifolia* Root, BHA: Butylated Hydroxyanisole, BHT: Butylated Hydroxytoluene, TOC: α-Tocopherol

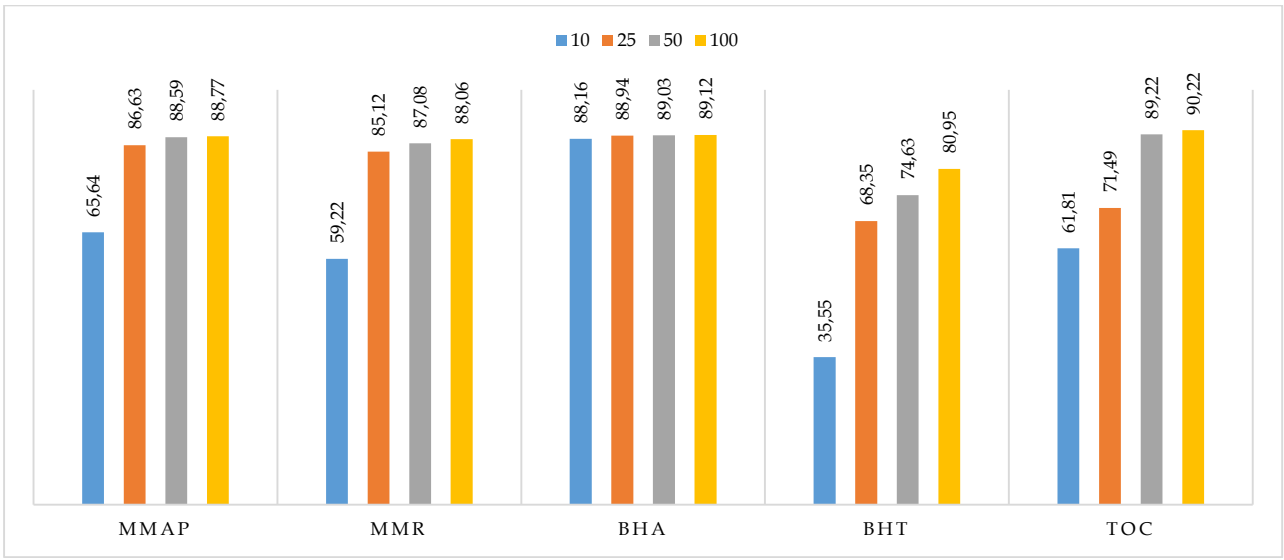

Figure 7. ABTS Cation Radical Scavening Activity Results (Concentration-Inhibition%), MMAP: *M. myrtifolia* Aerial Parts, MMR: *M. myrtifolia* Root, BHA: Butylated Hydroxyanisole, BHT: Butylated Hydroxytoluene, TOC: α-Tocopherol

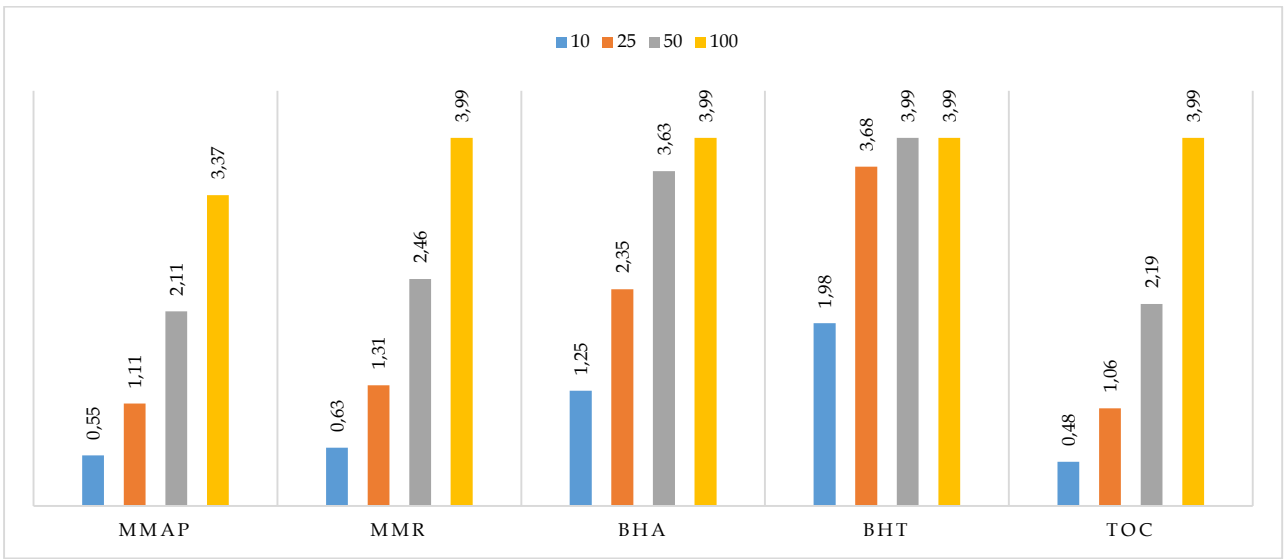

Figure 8. CUPRAC Activity Results (Concentration-Absorbance), MMAP: *M. myrtifolia* Aerial Parts, MMR: *M. myrtifolia Root*, BHA: Butylated Hydroxyanisole, BHT: Butylated Hydroxytoluene, TOC: α-Tocopherol

Micromeria species are renowned for being good sources of phenolic compounds, predominantly phenolic acids and flavonoids. As a consequence of this, their antioxidant properties are also well-established. The antioxidant activity of these extracts exhibits a dose-dependent relationship, indicating that higher concentrations generally lead to increased efficacy. Additionally, this activity is significantly influenced by the choice of solvents used for extraction, suggesting that different solvents can selectively extract various phytochemicals with antioxidant properties. This highlights the importance of solvent selection in optimizing the antioxidant potential of the extracts (Öztürk et al., 2011; Vladimir-Knežević et al., 2011; Formisano et al., 2014; Šamec et al. 2015; Abu-Gharbieh et al., 2016; Salameh et al., 2020; Sarikurkcu et al., 2020b; Adjdir et al., 2021; Mladenova et al., 2021; Sadeq et al., 2021; El Kamari et al., 2024). In the current study, the strong antioxidant activity of the extracts can be attributed to its rich composition of bioactive compounds. Nicotiflorin, the most abundant constituent, is a known radical scavenger with significant antioxidant properties (Patel, 2022). Rosmarinic acid, a well-established antioxidant, likely contributes through free radical scavenging and metal chelation. Other compounds such as quinic acid, caffeic acid, and naringenin further enhance the antioxidant activity due to their phenolic structures (Ersoy et al., 2022). The combined presence of these potent antioxidants highlights the efficacy of the studied extracts against oxidative stress.

The enzyme inhibitory activities of *M. myrtifolia* aerial parts and root extracts were assessed through acetylcholinesterase, butyrylcholinesterase, tyrosinase, urease, and α-glucosidase inhibition activity assays. The results are presented in Table 3.

Table 3. Enzyme Inhibitory Activity Results of the Studied Extracts

	Inhibition (%)				
Samples	AChE[a]	BChE[a]	Tyrosinase[a]	Urease[a]	α-glucosidase[b]
M. myrtifolia aerial parts	NA	NA	NA	NA	73.03±0.16
M. myrtifolia roots	NA	44.39±1.40	NA	NA	47.06±0.41
Galanthamine*	72.50±0.28	76.51±0.81	-	-	-
Kojic acid*	-	-	95.26±0.23	-	-
Tiyourea*	-	-		88,61±1,16	-
Acarbose*	-	-			81.53±0.50

a: The results are presented as the mean and standard deviation of three parallel measurements at a concentration of 200 µg/mL.

b: The study was conducted at a concentration of 2 mg/mL

*: Standard compounds

NA: Not Active

AChE: Acetylcholinesterase, BChE: Butyrylcholinesterase

In terms of anticholinesterase activity, only root extract of *M. myrtifolia* exhibited moderate inhibitory activity against butyrylcholinesterase with 44.39±1.40% at 200µg/mL concentration. Aerial parts extract was not active against acetylcholinesterase or butyrylcholinesterase. Regarding previous studies on the subject, in Öztürk et al. (2011)'s study, anticholinesterase activity was assessed in the extracts of *M. cilicica* in comparison to that of galantamine. Among the studied extracts prepared with different solvents, only the acetone extract demonstrated moderate inhibitory activity against butyrylcholinesterase, whereas others were not active at all. In Vladimir-Knežević et al. (2014)'s study, a total of 26 wild-growing species from the Lamiaceae family were tested for their anti-acetylcholinesterase potential. Four *Micromeria* species, namely *M. croatica*, *M. graeca*, *M. juliana*, and *M. thymifolia* were included in the study. Accordingly, only *M. graeca* exerted moderate-to-weak acetylcholinesterase inhibitory activity, while the other studied *Micromeria* species did not achieve acetylcholinesterase inhibition. Regarding current study, the lack of acetylcholinesterase inhibitory activity may be due to the lack of specific compounds that are shown to possess acetylcholinesterase inhibitory activity potential. Speaking of which, flavonoids such as quercetin, quercitrin, hyperoside, and rutin were found to be much more effective compared to phenolic acids in terms of anti-acetylcholinesterase activity (Orhan et al., 2006; Ersoy et al., 2020). The aerial parts of *M. myrtifolia* were found to contain only quercetin with 8.19±0.44 µg/g extract among the effective compounds. Rutin and quercitrin were not detected in the extracts.

Both extracts were found to be effective against the α-glucosidase enzyme. Compared to the standard molecule acarbose (81.53±0.50%), the aerial parts extract showed strong inhibitory activity with 73.03±0.16% and the roots extract demonstrated moderate inhibitory activity with 47.06±0.41% at 2 mg/mL concentration. In managing diabetes, controlling blood glucose levels after meals is crucial. One effective approach is through the inhibition of α-glucosidase enzymes, which play an essential role in carbohydrate digestion. Located on the brush-border membrane of intestinal cells, these enzymes catalyze the breakdown of oligosaccharides by hydrolyzing α-glycosidic bonds, releasing glucose molecules for absorption into the bloodstream. By slowing this process, α-glucosidase inhibitors help reduce the rapid rise in blood glucose that typically follows a meal (Lawag et al., 2012). Aleixandre et al. (2022) aimed to understand the role of phenolic compounds in alpha-glucosidase inhibition. Accordingly, the most potent molecules were phenolic acids, caffeic and protocatechuic acids in particular. Since the studied extracts were found to contain

these compounds, the activity is considered to be due to these molecules. They also noted that phenolic acids containing more than one hydroxyl group demonstrated the lowest IC_{50} values against α-glucosidase.

Neither the aerial parts nor the root extracts were found to inhibit tyrosinase and urease enzymes.

4. Conclusions

Micromeria species are recognized as valuable sources of biologically active compounds and highly regarded as medicinal plants within Türkiye. This study provided compelling evidence that extracts from the aerial parts and roots of *M. myrtifolia* are particularly rich in phenolic compounds, with exceptional levels of nicotiflorin. Furthermore, these extracts demonstrated notable antioxidant activity and anti-glucosidase inhibitory effects that are worth mentioning, highlighting their potential as therapeutic agents. The findings underscore the importance of *M. myrtifolia* not only as a traditional remedy but also as a candidate for developing functional foods or nutraceuticals aimed at managing oxidative stress and diabetes. Further research in this area will enhance our understanding of the medicinal properties of *M. myrtifolia*, paving the way for its application in diabetes management and other health-related interventions.

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Research Article/Araştırma Makalesi

Effects of Black Carrot Juice on Liver Lipid Peroxidation and Antioxidant Enzymes in Acutely Exercised Rats

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Abstract: In this study, the effects of black carrot juice on acutely exercised rat liver tissues were investigated. Rats were formed into Control (C), Black carrot juice (S), Acute exercise (E) and Black carrot juice + Acute exercise (SE) groups. The acute exercise program was applied on a treadmill device. Malondialdehyde (MDA) and Glutathione (GSH) levels were examined in the rat liver tissues obtained at the end of the experimental process, while Glutathione reductase (GR), glutathione-S-transferase (GST) and Carboxyl esterase (Ces) enzyme activity levels were measured. While MDA, GST and Ces enzyme activity levels in the E group increased compared to the K group, GR and GSH levels decreased compared to the K group. GST and Ces enzyme activities in the S group increased compared to the K group. While GSH level in the SE group increased compared to the E group, GST enzyme activity and MDA levels decreased. As a result, we think that black carrot juice prevents lipid peroxidation and has a supportive effect on the antioxidant enzyme system on the liver tissues of rats subjected to acute exercise.

Keywords: Exercise, black carrot, lipid peroxidation, antioxidant enzyme.

Akut Egzersiz Yaptırılan Sıçanlarda Karaciğerlerindeki Lipit Peroksidasyon ve Antioksidan Enzimleri Üzerine Siyah Havuç Suyunun Etkileri

Öz: Bu çalışmada, akut egzersiz yaptırılan sıçanların karaciğerlerinin dokuları üzerine siyah havuç suyunun etkileri araştırıldı. Sıçanlar Kontrol (K), Siyah havuç suyu (S), Akut egzersiz (E) ve Siyah havuç suyu + Akut egzersiz (SE) grupları oluşturuldu. Akut egzersiz programı treadmill cihazında uygulamalar yapıldı. Deneysel süreç sonunda elde edilen sıçan karaciğer dokularında malondialdehit (MDA) ve Glutatyon (GSH) düzeyleri incelenirken, Glutatyon redüktaz (GR), Glutatyon-S-transferaz (GST) ve Karboksil esteraz (Ces) enzim aktivite düzeyleri ölçüldü. E grubu MDA, GST ve Ces enzim aktivite düzeyleri K grubuna göre artarken, GR ve GSH düzeyleri ise K grubuna göre azaldı. S grubu GST ve Ces enzim aktiviteleri K grubuna göre arttı. SE grubu GSH düzeyi E grubuna göre artarken, GST enzim aktivite ve MDA düzeyleri azaldı. Sonuç olarak, akut egzersiz uygulanan sıçanların karaciğer dokuları üzerine siyah havuç suyunun lipid peroksidasyonu engellediği, antioksidan enzim sistemini destekleyici etkisinin olduğunu düşünmekteyiz.

Anahtar kelimeler: Egzersiz, siyah havuç, lipit peroksidasyon, antioksidan enzim.

1. Giriş

İskelet kasları tarafından üretilen enerjinin harcanmasını gerektiren bedensel hareketlere fiziksel aktivite denir. Fiziksel aktivite kas ve eklem hareketliliğini korur, eklem dayanıklılığını geliştirir, dokularda oksijen ve enerji miktarını artırır. Bu durum, metabolizmada koruyucu etkilere neden olmaktadır. Düzensiz yapılan egzersizler sonucunda meydana gelen metabolik son ürünler dokular üzerine olumsuz etkiler yapmaktadır. Uzun süreli olmayan yoğun egzersiz programlarından olan akut egzersiz, alışkın olmayan insan ve hayvanlarda tek seferde veya kısa süreli egzersiz uygulamalarındandır. Akut egzersiz metabolizmada oksidatif, stresi artırarak kas hasarlarına neden olmaktadır. Ayrıca, metabolizmada serbest radikal düzeyleri artmaktadır. Bunun sonucu olarak, hücre içerisinde enzimatik ve enzimatik olmayan antioksidan sistemler olumsuz etkilenmektedir (Halliwell, 1999; Taysi, et al.. 2008; Marques-Aleixo, et al., 2015; Allendorfer, & Arida. 2018).

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Siyah havuç içeriğinde, vitamin, mineral ve antisiyanin molekülleri yoğun olarak bulunmaktadır. Ayrıca sinnamik asit, flavonol ve kafeik asit gibi birçok antioksidan molekül vardır. Siyah havucun farmakolojik etkilerinde antikanserojen, antitoksik, antioksidan, antiflamatuar ve nörodejerenatif etkiler bulunmaktadır (Kammerer, et al., 2004; Metzger, et al., 2008; Wang. & Stoner, 2008; Sun, et al., 2009; Poudyal, et al., 2010). İnsanlar günlük hayatlarında akut egzersize maruz kaldığı gibi, aktif spor yapan bireylerin de egzersize maruz kaldığı bilinmektedir. Bu çalışmada siyah havuç suyunun akut egzersiz uygulanan sıçan karaciğer dokularındaki lipit peroksidasyon ve antioksidan enzim sistemleri üzerine etkilerinin araştırılması amaçlanmıştır.

2. Materyal ve Metot

2.1. Deneysel Aşama

Çalışmanın etik raporu, Adıyaman Üniversitesi Yerel Etik Komitesi tarafından onaylandı (İzin Numarası: 2024/40).

Deneyde kullanılan sıçanların, bakımı ve kullanımı ilgili ulusal ve uluslararası yasalara ve politikalara uygun şekilde yapıldı. Yirmi sekiz adet yetişkin 3 aylık erkek *Sprague Dawley* sıçan (vücut ağırlığı 250 ± 10 g) Adıyaman Üniversitesi Deney Hayvanları Üretim Uygulama ve Araştırma Merkezinden temin edildi. Sıçanlar, sabit sıcaklıkta (21 ± 1°C) ve nemde (%55 ± 5) standart bir ışık/karanlık programında (12 saat ışık/12 saat karanlık döngüsü) peletlenmiş yem ve taze musluk suyuna serbestçe erişebilecekleri şekilde muhafaza edildi. Sıçanlar rastgele Kontrol (K), Siyah havuç suyu grubu (S), Akut egzersiz grubu (E) ve Siyah havuç suyu + Akut egzersiz (SE) (her grupta n = 7) olmak üzere dört gruba ayrıldı. K grubu sıçanlara içme suyu verildi. Yerel pazardan alınan taze siyah havuçlar musluk suyunda yıkandı ve meyve sıkacağında sıkılarak sıçanlara verildi. Siyah havuç suyu 4 ml/kg dozda oragastrik gavaj yöntemiyle uygulaması yapıldı (Gülru Esen et al., 2021). Akut koşu egzersizinde sıçanlar, iki haftalık alıştırmadan sonra üç fazda uygulama işlemine tabi tutuldular. Birinci haftada sıçanların koşu bandında günde 10 dakika, haftada 3 gün (10 m/dk hız, % 0 eğim) koşturuldu. İkinci haftada sıçanların günde 10 dakika, haftada 3 gün (20 m/dk hız, % 0 eğim) koşturulması sağlandı. Alıştırma protokolünden yirmi dört saat sonra sıçanlara akut egzersiz oluşturmak için 3 aşamalı koşu bandı koşusu yaptırıldı. Birinci aşamada (dk 0-5): sıçanlar 15 m/dk hızla ve % 0 eğimle koşturuldu. İkinci aşamada (dk 5-10): sıçanlar 23 m/dk hızla ve %0 eğimle koşturuldu. Üçüncü aşamada (dk 10-60): sıçanlar 25 m/dk hızla ve %5'lik bir eğimle koşturuldu. Egzersiz yapılmayan gruplardaki sıçanlar (K ve S grupları), 60 dk boyunca hareket etmeyen koşu bandı üzerine yerleştirildi ve böylece koşu bandının kendisi tarafından tetiklenen potansiyel kullanım ve çevre streslerine maruz bırakıldı (Ascensao, et al., 2011). Deneysel uygulamalardan sonra sıçanlara servikal dislokasyon uygulanarak onların karaciğer dokularından elde edilen doku örneklerinde biyokimyasal işlemler gerçekleştirildi. Dokular -80 ^{o}C de deneysel sürece kadar saklandı.

2.2. Karaciğer Dokusu Homojenizasyonu

Karaciğer örneklerini homojenleştirmek için 0,15 M KCl, 1 mM EDTA ve 1 mM DTT içeren buzla soğutulmuş 0,1 M K-fosfat tamponu, toplam doku ağırlığının (w/v) 1:4 oranında kullanıldı. Homojenizasyon, Heidolph RZ 2021 markalı bir homojenizatörle gerçekleştirildi. Homojenatlar, Hettich ROTINA 420 R santrifüjü kullanılarak 4 ^{o}C derecede 30 dakika boyunca 15000 g'de santrifüjlendi. Santrifüjlemeden sonra elde edilen üst sıvı faz enzim aktivitesi için kullanıldı. MDA ve redükte GSH seviyeleri ile Ces, GST ve GR enzim aktiviteleri mikroplaka okuyucu sistemi (ThermoTM Varioskan Flash, Thermo Scientific) kullanılarak uygun dalga boylarında spektrofotometrik olarak ölçüldü.

2.3. GST Enzim Aktivitesinin Ölçümü

GST aktivite ölçümü, Habig et al. (1974) tarafından geliştirilen ve mikro plaka okuyucu sistemine uyarlanan yöntemin değiştirilmiş bir versiyonu kullanılarak yapıldı. Karışım 100 mM K-fosfat tamponu (pH 6,5), 1 mM GSH (kofaktör olarak kullanıldı), 10 mM 1-kloro-2,4-dinitrobenzen (CDNB, substrat olarak kullanıldı) ve üst sıvıdan oluşuyordu. Ölçüm sırasında sırasıyla 10 mikrolitre üst sıvısı, 100 mikrolitre fosfat tamponu + 100 µl GSH karışımı ve son olarak CDNB mikro plaka kuyularına pipetlendi. Reaksiyon sırasında tüketilen GSH'nin azalmasına bağlı olarak CDNB miktarındaki azalmayı ifade eden absorbans değişiklikleri 25 °C'de 3 dakika boyunca 344 nm'de ölçüldü ve özgül GST aktivitesi nmol dak^{-1}g protein^{-1} olarak kaydedildi.

2.4. Ces Enzim Aktivitesinin Ölçümü

Ces aktivitesi, Santhoshkumar ve Shivanandappa (1999) tarafından mikro plaka okuyucusu için açıklanan prosedürün değiştirilmiş versiyonu kullanılarak belirlendi. Reaksiyon çözeltisi 5 µL süpernatant ve 0,1 mM 250 µL pH 7,4 trizma tamponundan oluşturuldu. Mikro plaka kuyularına pipetlenen reaksiyon karışımı 25 °C'de 3 dakika inkübe edildi. Reaksiyon, kuyulardaki reaksiyon çözeltisine 5 µL 0,5 mM p-nitrofenol asetat (PNPA) eklenerek başlatıldı. Ces tarafından substrat olarak p-nitrofenol asetat kullanılması nedeniyle reaksiyon sırasında salınan p-nitrofenol, 25 °C'de 2 dakika boyunca 405 nm'de izlendi. Spesifik Ces aktivitesi nmol dak^{-1}g protein^{-1} olarak hesaplandı.

2.5. GR Enzim Aktivitesinin Ölçümü

GR enzim aktivitesi için reaksiyon karışımı 0.1 mM, 150 µl 5,5'-dithiobis (2-nitrobenzoik asit) (DTNB), 1.2 mM, 20 µl NADPH ve 20 µl örnek içermektedir.20 µl, 3.25 mM GSSG'nin ilavesi ile reaksiyon başlatılmıştır. Bütün çözeltiler, 1 mM EDTA içeren, 0.1 M potasyum fosfat tamponunda (pH 7.5) hazırlandı. Reaksiyon ilerledikçe GSSG'den GSH oluşumu nedeniyle azalan DTNB miktarı oda sıcaklığında 3 dakika süre ile 405 nm'de izlendi. Spesifik GR aktivitesi nmol dak^{-1}g protein^{-1} olarak hesaplandı (Cribb, et al., 1989).

2.6. MDA ve GSH Düzeyleri Ölçümü

Lipid peroksidasyonun ürünü olan MDA'nın analizi Placer et al. (1966) tarafından açıklandığı şekilde gerçekleştirildi. Reaksiyon karışımı, 1,5 mL reaksiyon çözeltisine 500 µL homojenat eklenerek, eşit hacimde %15 trikloroasetik asit, %0,375 tiyobarbiturik asit, 0,25 N HCl (1:1:1, w/v) karıştırılarak taze olarak hazırlandı. Reaksiyon karışımı, 100 °C'de 30 dakika su banyosunda ısıtıldı. Karışım oda sıcaklığına soğutulduktan sonra, santrifüjleme 15000 g'de 15 dakika gerçekleştirildi. Daha sonra, süpernatant örnekleri mikro plaka kuyularına aktarılarak, reaksiyon sırasında oluşan MDA'ya bağlı absorbans değişiklikleri 532 nm'de kaydedildi. MDA seviyeleri (nmol/g ıslak doku ağırlığı) olarak ifade edildi.

GSH seviyesi, redükte GSH'nin 5.5-dithiobis 2-nitro-benzoik asit (DTNB) ile reaksiyonuyla oluşan bileşiğin 412 nm'deki absorbans değerinin ölçülmesiyle belirlendi (Moron, et al., 1979). Redükte GSH seviyesi nmol mg^{-1} protein^{-1} olarak ifade edildi.

2.7. Total Protein Analizi

Üst sıvı örneklerindeki toplam protein içeriği Bradford 1976 kolorimetrik yöntemi ve standart olarak sığır serum albümini (BSA) kullanılarak belirlendi (Bradford, 1976). Sonuçlar miligram protein olarak ifade edildi. Tüm analizler üç kez gerçekleştirildi.

2.8. İstatistiksel Analiz

Sıçanların karaciğerinin dokularındada tespiti yapılan biyokimyasal parametrelerin karşılaştırılması için SPSS 20,0 programı kullanıldı. Biyokimyasal parametrelerin

istatistiksel karşılaştırması Tek Yönlü Varyans Analizi kullanılarak yapıldı ve ardından Tukey-HSD testi uygulandı. Veriler, ortalama ± SEM (ortalama standart hata) olarak ifade edildi.

3. Bulgular

Sıçan karaciğer dokuları biyokimyasal parametreleri Tablo 1'de gösterilmiştir. E ve SE grupları MDA düzeyi K grubuna göre arttı ($p<0.001$, $p<0.01$). E grubu MDA düzeyine göre SE grubunda azalma tespit edildi ($p<0.001$). K grubu GSH düzeyine göre E ve SE gruplarında azalma gözlendi ($p<0.001$). SE grubu GSH düzeyi E grubuna göre arttı ($p<0.05$). K grubu GST enzim aktivitesi düzeyine göre E, S ve SE gruplarında önemli istatistiksel artışlar gözlendi ($p<0.001$). E grubu GST enzim aktivitesi düzeyine göre SE grubunda azalma tespit edildi ($p<0.01$). K grubu Ces enzim aktivite düzeyine göre S, E ve SE gruplarında önemli artışlar gözlendi ($p<0.01$, $p<0.001$). K ve S grupları GR enzim aktivelerinde istatistiksel fark gözlenmezken, K grubu GR enzim aktivitesine göre E ve SE gruplarında önemli azalma gözlendi ($p<0.001$).

Tablo 1. Sıçan karaciğer dokuları biyokimyasal parametreler

Table 1. Biochemical parameters of rat liver tissues

Gruplar	MDA	GSH	GST	Ces	GR
K	9.51 ±0.89	0.25 ±0.007	64.46 ±2.84	2294.96 ±79.23	12.47 ±0.66
S	10.13 ±1.12^{z}	0.24 ±0.004^{z}	79.30 ±2.51^{cz}	2767.68 ±64.76^{bx}	11.08 ±0.74^{z}
E	19.42 ±2.75^{c}	0.17 ±0.005^{c}	94.70 ±2.34^{c}	3108.89 ±101.79^{c}	4.81 ±0.24^{c}
SE	13.79 ±1.56^{bz}	0.21 ±0.006^{cx}	81.48 ±1.69^{cy}	2927.87 ±72.24^{c}	5.5 4±0.44^{c}

K grubuna göre istatistiksel karşılaştırma: a: $p<0.05$; b: $p<0.01$; c: $p<0.001$

E'ye göre istatistiksel karşılaştırma: x: $p<0.05$; y: $p<0.01$; z: $p<0.001$

4. Tartışma ve Sonuç

Bu araştırmada sıçanlara uygulanan akut egzersize karşı siyah havuç suyunun lipid peroksidasyon ve bazı enzimler üzerine etkileri araştırıldı.

Egzersizin yoğunluğuna bağlı olarak metabolizmada olumlu ve olumsuz etkiler ortaya çıkmaktadır (Gradari, et al., 2016; Guerreiro, et al., 2016). Egzersizin planlı olarak yapılması durumunda metabolizmaya birçok faydası vardır. Bu etkiler içerisinde vücut ağırlığının ve yağının azalması, kemik yoğunluğunun artması, kardiyovasküler, obezite ve kanser hastalıklarını azaltması bulunmaktadır (Vina, et al., 2000; Selman, et al., 2002; Naderi, et al., 2015; Emami, et al., 2016). Ancak; aşırı yapılan egzersizin metabolizmada birçok olumsuz etkisi bilinmektedir. Bunlar arasında en önemlisi, hücre mitokondrisinde oksijen tüketimi artarak serbest radikal üretiminin artırılmasıdır. Bu radikaller hücrede lipitler, proteinler ve DNA hasarlarını artırmaktadır (Emami, et al., 2016; Gradari, et al., 2016). Araştırmada, akut egzersiz uygulanan sıçan karaciğer dokularında lipit peroksidasyon ürünü olan MDA'nın arttığı, GSH düzeyinin ve GR enzim aktivitesinin azaldığı tespit edilmiştir. Egzersiz koşularında koşu hızının seviyesi çok önemlidir. Bu alanda yapılan önemli bir çalışmada, farklı koşu hızlarının oksidatif stres düzeylerine etkileri araştırılmıştır. 36 erkek *Wistar* sıçanları 10,13,14,5,16 ve 17,5 m/dk yoğunluğunda koşu uygulaması yapılan araştırmada sonucunda <16 m/dk. hızlarda egzersiz yapan grupların karaciğer dokuları katalaz enzim (CAT) aktivitesinin ve kaslarda GST aktivitesini artırdığı, GSH seviyesini her iki dokuda da azalttığını rapor etmişlerdir. <16 m/dk. hızlarda iskelet kasındaki antioksidan sistem yanıtının adaptasyonu aktive ettiğini, ancak yüksek hızlarda oksidatif strese neden olduğu belirtilmiştir (Mousavi, et al., 2020). Çalışmada uygulanan protokole göre maksimum 25m/dk. hızda koşturulan sıçanların karaciğer dokularındaki azalan GSH düzeyi ve artan MDA düzeyleri çalışma protokolünün bu literatürle uyumlu olduğunu göstermektedir. Akut egzersiz sonucunda serbest radikal düzeylerinde artışlar olmaktadır. Ancak bu artışı hücre sisteminde savunucu antioksidan enzimler inhibisyona uğratırlar. Özellikle süperoksit dismutaz (SOD), GST, CAT, GR ve glutatyon peroksidaz (GPx) gibi enzimlerin yanı sıra GSH gibi antioksidan moleküller devreye girerek inhibisyon sağlarlar (Powers, ve Jackson, 2016; Carlos et al., 2019).

Ayrıca, yorucu yapılan egzersiz sonucunda dokularda MDA düzeyinde artışlar ve GSH düzeylerinde de azalmalar rapor edilmiştir (Berzosa, et al., 2011; Radak, et al., 2014; Toval, et al., 2017). Akut yorucu egzersiz uygulanan sıçan karaciğer dokularında MDA ve tiyabarbitürük asit reaktif maddelerinin düzeylerinde artışlar, GSH, SOD, CAT enzim aktivitelerinde azalmalar bildirilmiştir (Taysi, et al., 2008; Huang, et al., 2009; Korivi et al., 2012; Huang, et al., 2013; Qi, et al., 2014). Akut yorucu egzersiz yaptırılan sıçan karaciğer dokuları üzerine değişik dozajlarda tartar karabuğday ekstraktı takviyesinin doz artırımına bağlı olarak MDA düzeylerinde azalma, SOD, CAT, GPx ve GR enzim aktivitelerinde ise önemli düzeyde artışlar rapor edilmiştir (Wei, et al., 2012). Akut yoğun egzersize maruz kalan sıçan karaciğer dokuları üzerine brokoli özütünün etkinliği araştırmasında, egzersiz grubu GST, GR ve GPx enzim aktivite düzeyi ile kontrol grubu arasında anlamsal fark olmadığı tespit edilmiştir. Ancak, egzersiz + brokoli grubunda brokoli diyetinin etkisiyle GST, GR, GPx aktivitelerinde artışlar tespit edilmiştir. Ayrıca, akut egzersiz uygulanan grubun CAT enzim aktivitesinin azaldığı, bu azalmayı brokoli diyetinin düzelttiği bildirilmiştir (Cardenia, et al., 2017). Yapılan başka bir araştırmada, sıçanlar üzerine uygulanan yüzdürme egzersizi sonucunda karaciğer dokularında SOD ve CAT enzim aktivitelerinde artışlar tespit edilirken, GSH düzeylerinde azalma tespit edilmiştir (Radu, et al., 2010). GSH molekülü GPx ve GST enzim aktiviteleri için substrat görevini üstlenir. GSH molekülünün azalması oksidatif stresin belirteci olarak kabul edilir (Powers,&Malcom, 2008; Olguin, et al., 2019). Hücrede GSH molekülü düzeyinin azalması, artan oksidatif stres ile artan GST enzim aktivitesinden kaynaklanmaktadır. Araştırmamızda, egzersiz yaptırılan sıçan karaciğer dokularında azalan GSH düzeyi yukarıda belirtilen araştırmalarla uyumlu olduğu görülmektedir (Bejma, J & Ji, 1999; Radu, et al., 2010; Vukovic, et al., 2014). Çalışmada, siyah havuç suyu uygulanan egzersiz grubunun MDA düzeyi siyah havuç suyu etkisiyle egzersiz grubundan az çıktığı tespit edildi. Siyah havuç suyu uygulanan egzersiz grubu GSH düzeyi ise egzersiz grubuna göre arttığı gözlendi. Egzersiz grubu GR enzim aktivitesi diğer gruplara göre en düşük düzeyde tespit edilmiştir. Ayrıca, egzersiz grubu GST ve Ces enzim aktivite düzeyleri diğer

gruplara göre en yüksek seviyede gözlenirken, siyah havucun etkisiyle kombinasyonlu grupta bu enzim aktivite artışları azalmıştır.Siyah havuç suyun içeriğinde antioksidan etkinliğinin yüksek olduğu bilinen antosiyanidin molekülleri mevcuttur (Ulusoy, & Tamer, 2019). Bu moleküllerin antimikrobiyal, antiobezite, antikanser ve kardiyovasküler hastalıklara karşı etkinlikleri vardır (Mizgier, et al., 2016; Khoo, et al., 2017). Çalışmada, egzersiz grubu Ces ve GST enzim aktivite düzeylerinin diğer gruplara göre yüksek seviyede çıktığı gözlenmiştir. Bu sonuçlarımızın, akut egzersiz uygulaması sonucunda hücrede artan radikallerin inhibisyonu için bu enzimlerin aktivasyonun artmasına neden olduğunu düşünmekteyiz. Çünkü bu enzimler hücrede detoksifikasyon görevini üstlenmektedirler. Ancak, siyah havuç uygulaması sonucunda akut egzersizin artırdığı Ces ve GST enzim aktivitelerini azalttığı tespit edildi. Bu azalmayı, siyah havucun antioksidan etkilerinden kaynaklandığı söylenebilir. Yapılan bir araştırmada siyah havuç suyunun akrilamid nörotoksisitesine karşı düzeltici etkisi olduğu rapor edilmiştir. Sıçan dokularında akrilamidin lipit peroksidasyonu ve GST enzim aktivitesini artırırken, GSH düzeyini azalttığı rapor edilmiştir (Esen, et al., 2021). Yapılan literatür araştırmalarında, akut egzersiz çeşitlerinin tamamında Ces enzim aktivitesinin çalışılmadığı gözlenmiştir. Bu enzim ilaç ve pestisit gibi bileşiklerin metabolizması için çok önemlidir. Ayrıca, organofosfor ve karbamat pestisitleri Ces enzimlerinin inhibisyonuna neden olur (Di, 2019). Akut egzersiz insanların günlük hayatı içerisinde sıkça karşılaştığı bir durumdur. Gerek sporcular gerekse sedanter bireyler akut egzersiz olayı ile daha sıkça karşılaşmaktadır. Çalışmada, akut egzersizin sıçan karaciğer dokularında yüksek lipit peroksidasyona neden olduğu, GSH ve GR düzeylerini azalttığı tespit edildi. Detoksifikasyon enzimlerden GST ve Ces enzim aktivite düzeyleri akut egzersizin etkisiyle arttığı gözlendi. Siyah havuç suyunun akut egzersizin oluşturduğu yüksek lipit peroksidasyonu azaltarak, antioksidan enzim sistemindeki enzimlere pozitif etkiler sağladığı tespit edildi. Sonuç olarak, akut egzersizin oluşturduğu olumsuz etkilere karşı siyah havuç suyunun düzeltici etkileri olabileceğini düşünmekteyiz.

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Therapeutic Potential of *Quercus ithaburensis* subsp. *macrolepis* Fruit Extract in Streptozotocin-Nicotinamide-İnduced Type 2 Diabetic Rats

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Abstract: In this study; the effect of *Quercus ithaburensis* subsp. macrolepis fruit extract (QIFE) on blood glucose and oxidant-antioxidant systems in streptozotocin (STZ)-nicotinamide-induced type 2 diabetic rats was investigated. Type 2 diabetes was induced in rats by intraperitoneal injection of STZ (65mg/kg)-Nicotinamide (45 mg/kg). Rats were given 535mg/kg QIFE fruit extract in their drinking water for 21 days. Rats were divided into four groups; Control (C), Control+QIFE (C+QIFE), Diabetes (D), and Diabetes+QIFE (D+QIFE). Plasma and tissue malondialdehyde (MDA) levels were measured by spectrophotometry. Whole blood glutathione peroxidase (GSH-Px), serum superoxide dismutase (SOD) enzyme levels, serum paraoxonase (PON), and arylesterase (ARE) enzyme activities were determined using commercial kits. Serum insulin levels and blood glucose were evaluated using a Rat ELISA Kit and glucometer, respectively. Also, the autoanalyzer was used to assess the lipid profile. While blood sugar and serum total cholesterol (TC) levels showed a statistically significant decrease in the C+ QIFE and D+ QIFE groups (C and D groups, respectively), serum insulin levels showed a statistically significant increase in the D+QIFE group compared to the D group. In the D+QIFE group, a statistically significant increase was observed in PON and ARE enzyme activities compared to the D group, but in the C+QFE group, a significant increase was found in whole blood GSH-Px and serum SOD levels compared to the C group. A statistically significant decrease was detected in plasma, heart, muscle, and liver tissue MDA levels in the D+QIFE group compared to the C group. As a result, it was concluded that *Q. ithaburensis* fruit extract has anti-hyperglycemic, anti-hyperlipidemic effects, strengthens the antioxidant system, and is a good phytotherapeutic agent that prevents/improves metabolic processes and related complications related to diabetes mellitus.

Keywords: Antioxidant, lipid peroxidation, phytotherapeutic agents, oxidative stress.

Streptozotosin-Nikotinamid ile Oluşturulmuş Tip 2 Diyabetik Sıçanlarda *Quercus ithaburensis* subsp. *macrolepis* Meyve Ekstraktının Terapötik Potansiyeli

Öz: Bu çalışmada; *Quercus ithaburensis* subsp. macrolepis meyve ekstraktının streptozotosin (STZ)-nikotinamid ile oluşturulan tip 2 diyabetli sıçanlarda kan glikozu ve oksidan-antioksidan sistemler üzerindeki etkisi araştırıldı. Sıçanlarda tip 2 diyabet, STZ (65 mg/kg)-nikotinamidin (45 mg/kg) intraperitoneal enjeksiyonuyla oluşturuldu. Sıçanlara 21 gün boyunca içme sularına 535mg/kg *Q. ithaburensis* meyve ekstraktı verildi. Sıçanlar dört gruba ayrıldı; Kontrol (K), Kontrol+*Q. ithaburensis* meyve ekstraktı (K+QIFE), Diyabet (D), Diyabet+*Q.ithaburensis* meyve ekstraktı (D+QIFE). Plazma ve doku malondialdehit (MDA) düzeyleri spektrofotometre ile ölçüldü. Tam kan glutatyon peroksidaz (GSH-Px), serum süperoksit dismutaz (SOD) enzim düzeyleri, serum paraoksonaz (PON) ve arilesteraz (ARE) enzim aktiviteleri ticari kitlerde belirlendi. Serum insülin düzeyleri ve kan glikozu sırasıyla bir Rat ELISA Kiti ve glukometre ile değerlendirildi. Ayrıca, lipid profilini değerlendirmek için otoanalizör kullanıldı. Kan şekeri ve serum total kolesterol (TK) düzeyleri K+QIFE ve D+ QIFE gruplarında (sırasıyla K ve D grupları) istatistiksel olarak anlamlı bir azalma gösterirken, serum insülin düzeyleri D+QIFE grubunda D grubuna kıyasla istatistiksel olarak anlamlı bir artış gösterdi. D+QIFE grubunda PON ve ARE enzim aktivitelerinde D grubuna kıyasla istatistiksel olarak anlamlı bir artış gözlendi, ancak K+QFE grubunda tam kan GSH-Px ve serum SOD düzeylerinde K grubuna kıyasla anlamlı bir artış bulundu. D+QIFE grubunda K grubuna kıyasla plazma, kalp, kas ve karaciğer doku MDA düzeylerinde istatistiksel olarak anlamlı bir azalma tespit edildi. Sonuç olarak, *Q. ithaburensis* meyve ekstraktının antihiperglisemik, antihiperlipidemik etkiye sahip olduğu, antioksidan sistemi güçlendirdiği, diyabete bağlı metabolik süreçleri ve buna bağlı komplikasyonları önleyen/iyileştiren iyi bir fitoterapötik ajan olduğu sonucuna varıldı.

Anahtar kelimeler: *Antioksidan, lipid peroksidasyonu, fitoterapötik ajan, oksidatif stres.*

1. Introduction

From ancient times to the present, it is known that herbal medicines or their extracts are used to protect or treat health. It is known that plants are widely preferred by the public because they are natural and present a reduced risk of side effects compared to synthetic drugs. What is considered natural among people is the polyphenols (such as flavonoids, lignans, stilbenes, and phenolic acids) found in the structure of plants and have strong antioxidant properties (Shweta et al., 2021; Mithun et al., 2022). Polyphenolic compounds have many effects, such as antioxidant, anticancer, antimicrobial (Ahmed et al., 2016), antithrombotic (Mirza et al., 2019), and antidiabetic effects (Ngan et al., 2020). Today, the antidiabetic effect of polyphenols is one of the most researched areas and the mechanisms of action of approximately five hundred plants have been studied and established (Ramesh et al., 2017; Lin et al., 2018; Tarique et al., 2020).

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Type 2 diabetes (non-insulin-dependent diabetes) is one of the rapidly spreading chronic diseases in the world. Genetic and environmental factors are effective in the formation of this disease, and in both cases, heterogeneous insulin resistance and pancreatic beta-cell dysfunction are in question (Ralph et al., 2015). In addition, oxidative stress resulting from hyperglycemia, hyperlipidemia, obesity, and alterations in the antioxidant defense system contributes to the development of microvascular and macrovascular complications (Surapon, 2015; de Gaetano et al., 2018). Diabetes treatment is a life-long disease that needs to be followed closely. Therefore, it is necessary to consider that there are different strategies for the management of this disease and to evaluate it well. Among these, reducing and controlling postprandial hyperglycemia caused by carbohydrate intake is very common, especially in diabetes patients. For this reason, it is important to inhibit the catalytic activities of α-amylase and α-glucosidase which are effective in carbohydrate metabolism to control postprandial blood sugar (Bhandari et al., 2008; Jianwei et al., 2014). Another is preventing the formation of advanced glycation end products (AGEs) (Matsuda et al., 2003). Control of all of these is provided by synthetic inhibitory agents, but their side effects should not be ignored (Nissen et al., 2007; Neuman et al., 2012; Eugene et al., 2016). A further approach is to strengthen the antioxidant defense against oxidative stress in diabetes. In fact, our body creates the primary defense against oxidative stress through its antioxidant enzyme system, which includes enzymes like glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) (Pisoschi et al., 2015). Also, antioxidant vitamins (e.g., C, E, B1, and B6) and polyphenols, which are powerful antioxidants, play a crucial role in preventing and combating oxidative stress. Supplementing diabetic conditions with these vitamins and polyphenolscan help enhance our antioxidant defense (Sarandol et al., 2020; Tas et al., 2014; Tas et al., 2024; Zanza et al., 2019; Sen et al., 2010).

Quercus, one of the plants used in diabetic conditions, belongs to the Fagaceae family and is known as the oak tree. *Quercus* spp. consists of about 600 species worldwide, which produce some fruit commonly known as an acorn (Vinha et al., 2019). Acorn fruit is abundant in phenolic compounds (such as ellagic acid and gallic acid derivatives), flavonoids (quercetin, catechin, naringin), and tannins, as well as other unsaturated fatty acids such as linoleic, oleic, and palmitic acids (Rakic et al., 2006; Taib et al., 2020; Makhlouf et al., 2019, Ana et al., 2016). Additionally, acorns are rich in protein, fiber, and minerals and contain vitamins A and E (with tocopherols found in significant amounts) which support and strengthen their antioxidant structure (Özcan, 2007; Özcan, 2006; Özcan, 2005). *Quercus* species; bark, leaf, and fruit extracts have broad mechanisms of action such as antidiabetic (Yin et al., 2018) anti-inflammatory (Huang et al., 2016), anticancer (Zehra et al., 2019), neuroprotective (Gezici & Sekeroğlu, 2019), and antioxidant (Rakic et al., 2006).

There are many studies on the effects of *Quercus* species. However, no study has been found on the effects of *Q. ithaburensis* subsp. macrolepis (Kotschy) Hedge and Yalt fruit extracts on STZ-nicotinamide induced type 2 diabetic rats.

In this study, the effects of *Q. ithaburensis* subsp. macrolepis fruit extract on STZ-nicotinamide-induced type 2 diabetic rats were examined. To evaluate antioxidant mechanisms, serum PON and ARE activities, blood GSH-Px, and erythrocyte SOD levels were measured. Plasma and tissue MDA (heart, gastrocnemius muscle, liver, kidney) levels were measured to evaluate lipid peroxidation status. Also, blood glucose, serum insulin, and lipid levels were determined.

2. Material and Method

2.1. Experimental Design

Forty male Wistar albino rats, aged 3 months, were acquired from the Animal Center of Bursa Uludağ University (Ethic number 2018-04/11). The rats, grouped four per cage, were kept in an environment with a constant temperature of 25 ± 2°C and a 12-hour light/dark photoperiod throughout the experiment. During this period, the rats were given a standard pelleted diet and tap water.

The rats (n=10) were assigned to four distinct groups: Group 1: Normal control rats (C), Group 2: Control rats administered *Q. ithaburensis* fruit extract (C+QIFE), Group 3: STZ- Nicotinamide-induced diabetic rats (D), Group 4: STZ-nicotinamide-induced diabetic rats administered *Q. ithaburensis* fruit extract (D+QIFE).

2.2. Diabetes Induction

The STZ-nicotinamide diabetes model previously described by Masiello et al. (1998) was applied with a modified dosage. Rats were first injected with 45 mg/kg body weight of nicotinamide intraperitoneally (I.P.), and 15 minutes later, they were given a single intraperitoneal dose of 65 mg/kg body weight of STZ, prepared in citrate buffer (pH 4.5). Rats with blood glucose levels ≥200 mg/dL (measured with IME-DC blood glucose meters, Germany) 48 hours after injection were considered diabetic.

2.3. *Quercus ithaburensis* subsp. *macrolepis* Fruit Extract

Quercus ithaburensis subsp. macrolepis was collected from the skirts of Kaz Mountain, located to the north of Edremit Gulf, within the borders of Edremit District in Balıkesir Province, and *Q. ithaburensis* plant (above-ground part) extract was prepared by a commercial company (Kale Natural Bitkisel Ürünler, Edremit/Balıkesir). The QIFE extract came from the company in sterile dark glass bottles as 100mL/5g and we diluted it by pouring it into 400mL water. According to the water consumption per rat, 535mg/kg QIFE fruit extract was given to the drinking water of group II (C+QIFE) and group IV (D+QIFE) rats one week after STZ injection for 21 days. In our study, the *Q. ithaburensis* dose to be given to diabetic rats was evaluated as reference according to the administration doses of different Quercus species such as 25 mg/kg (Doğan et al., 2015), 200 and 800 mg/kg (Yin et al., 2018), 250 mg/kg, 500 mg/kg (Saini et al., 2012). The fluid and food intake of all groups of rats were measured daily, while body weight and blood sugar levels were measured weekly using an Abbott glucometer (USA).

2.4. Sample Preparation

Four weeks after the experimental procedure, blood

samples were taken from the rats by cardiac puncture under 3% isoflurane inhalation throughout the operation. Then, the heart, skeletal muscle (gastrocnemius muscle), liver, and kidney tissues were quickly removed, rinsed with a standard normal saline cold solution, and stored at -20°C. For plasma samples blood samples were collected into tubes containing EDTA and/or heparin. Plasma and serum samples were then centrifuged at 3000 rpm for 15 minutes and stored at -20°C until analyzed.

2.5. Determination of Biochemical Parameters

Serum lipid levels (TC, HDL-C, TG) were measured using an autoanalyzer (Abbott, Architect, USA). Serum insulin levels were determined using a Rat ELISA kit (Lab Science, E-EL-R2466, USA) and the results were expressed as ng/mL. In addition, serum SOD and blood GSH-Px levels were determined using a kit (YL Biotech, Shanghai) and the values were expressed as ng/mL. Serum PON1 and ARE enzyme activities were determined using a commercial kit and the units were expressed as U/L. (Rel Assay Diagnostics, Mega Tıp, Gaziantep, Türkiye). MDA levels of tissues (heart, skeletal muscle, liver, and kidney) were studied according to the method of Ohkawa et al. (1979) and the values were expressed as nmol MDA/mg tissue expressed. Also, according to the method of Young & Trimble (1991) plasma MDA concentration was determined. The results are expressed as nmol/mL.

2.6. Statistical Analysis

Statistical analyses were performed using SPSS 20.0 for Windows (SPSS, Chicago, IL), and the data are presented as the mean ± standard error of the mean (SEM). The Kruskal–the Wallis test followed by the Mann–Whitney U test was used. A level of $p<0.05$ was accepted as statistically significant.

3. Results

As presented in Table 1, food ($p<0.05$) and water intake ($p<0.01$), blood glucose ($p<0.01$), and total cholesterol (TC) levels ($p<0.05$) were significantly higher in group D rats compared to control rats, whereas a significant decrease in serum insulin levels was seen ($p<0.01$). D+QIFE group rats that were given QIFE fruit extract had a statistically significant decrease in food ($p<0.01$) and water intake ($p<0.05$), blood glucose ($p<0.01$), and TC levels ($p<0.05$) compared to group D rats, while a significant elevation in insulin levels was observed ($p<0.01$). Compared to group C, the increase in body weight in the C+QIFE group was not statistically significant. There was a significant increase in serum GSH-Px and SOD enzyme activities in the C+QIFE group compared to the C group ($p<0.01$ and $p<0.05$, respectively).

A significant reduction in blood glucose and TC levels ($p<0.05$) was detected in C+QIFE group rats compared to the control group rats. In the D group, SOD and blood GSH-Px levels were elevated ($p<0.05$) when compared to the C group rats but PON and ARE enzyme activities were found to be statistically lower ($p<0.01$). However, only PON and ARE enzyme activities were significantly higher in the D+QIFE group ($p<0.01$) compared to the D group. Additionally, in the D group rats, SOD and GSH-Px levels were significantly increased ($p<0.05$) compared to the C group rats (Table 2).

Table 1. Body weight, food and water consumption, serum glucose and insulin levels, and lipid profile of the study group

Group	C	C+QIFE	D	D+QIFE
Food intake (g/24s)	24.1 ± 1.3	22.8 ± 0.4	34.1 ± 0.5^{a*}	30.2± 0.46^{b**}
Water intake (mL/24s)	41 ± 1.3	45 ± 1.4	85 ± 1.3^{a**}	76 ± 2.3^{b*}
Body weight (g)	288 ± 7	300 ± 3.1	298 ± 4.5	289 ± 6
Glucose (mg/dL)	133.6± 3.2	118.5± 2.5^{a*}	294± 18^{a**}	278.4 ± 5.6^{b**}
Insulin (ng/mL)	1.7 ± 0.1	1.7 ± 0.2	0.6 ± 0.06^{a**}	1.02 ± 0.03^{b**}
TC (mg/dL)	60.2 ± 1.5	48 ± 1.7^{a*}	69.6± 1.8^{a*}	60± 2.6^{b*}
TG (mg/dL)	78.4 ± 2.2	73.5 ± 3.5	80.6 ± 3.6	79.5 ± 3.5
HDL-C (mg/dL)	50.8 ± 1.9	51.1± 1.3	54.5 ± 1.0	55.7 ± 1.6

TC: Total cholesterol, TG: Triglyceride, HDL-C: High-density lipoprotein cholesterol, C: Normal control rats, C+QIFE: Control rats with orally administered *Q. ithaburensis* fruit extract, D: Streptozotocin-Nicotinamid induced diabetic rats, D+QIFE: Diabetic rats with orally administered *Q. ithaburensis* fruit extract. Values are presented as the mean±SEM (standard error of the mean) for ten rats in each group, Statistical comparison: ^{a} C vs C+QIFE, ^{b} C vs D, ^{c} D vs D+QIFE, Statistical significance, * $p < 0.05$, ** $p < 0.01$

Table 2. Serum paraoxonase and arylesterase activities, superoxide dismutase, and blood glutathione peroxidase levels in rats.

Group	C	C+QIFE	D	D+QIFE
Blood GPX (ng/mL)	8.3 ± 1.4	12.8 ± 0.53^{a**}	11.8 ± 0.85^{a*}	13.14 ± 0.66
Blood SOD (ng/mL)	0.94 ± 0.17	1.59 ± 0.14^{a*}	1.28 ± 0.03^{a*}	1.31 ± 0.32
PON (U/L)	136.5 ± 8.5	144.7 ± 9.8	52.3 ± 3.5^{a**}	160.1 ± 9.5^{b**}
ARE (U/L)	140.1 ± 2.4	143.1 ± 1.3	59.7 ± 2.4^{a**}	148.7 ± 7.4^{b**}

PON: Paraoxonase, ARE: Arylesterase, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, Values are expressed as mean ± SEM (standard error of the mean) for ten rats in each group, Statistical comparison: ^{a} C vs C+QIFE ^{b} C vs D, ^{c} D *vs* D+QIFE, Statistical significance, * $p < 0.05$, ** $p < 0.01$, for abbreviations of study groups, see Table 1.

According to C group rats, plasma (Figure 1) and tissue MDA heart, musle, liver, kidney (Figure 2) levels were significantly higher in the D group rats ($p<0.01$). Plasma, heart, muscle ($p<0.01$), and liver ($p<0.05$) tissue MDA levels were significantly diminished in the D+QIFE group compared with the D group. However, the reduction in

plasma and tissue MDA levels in the C+QFE group, compared to the C group rats, was not statistically significant.

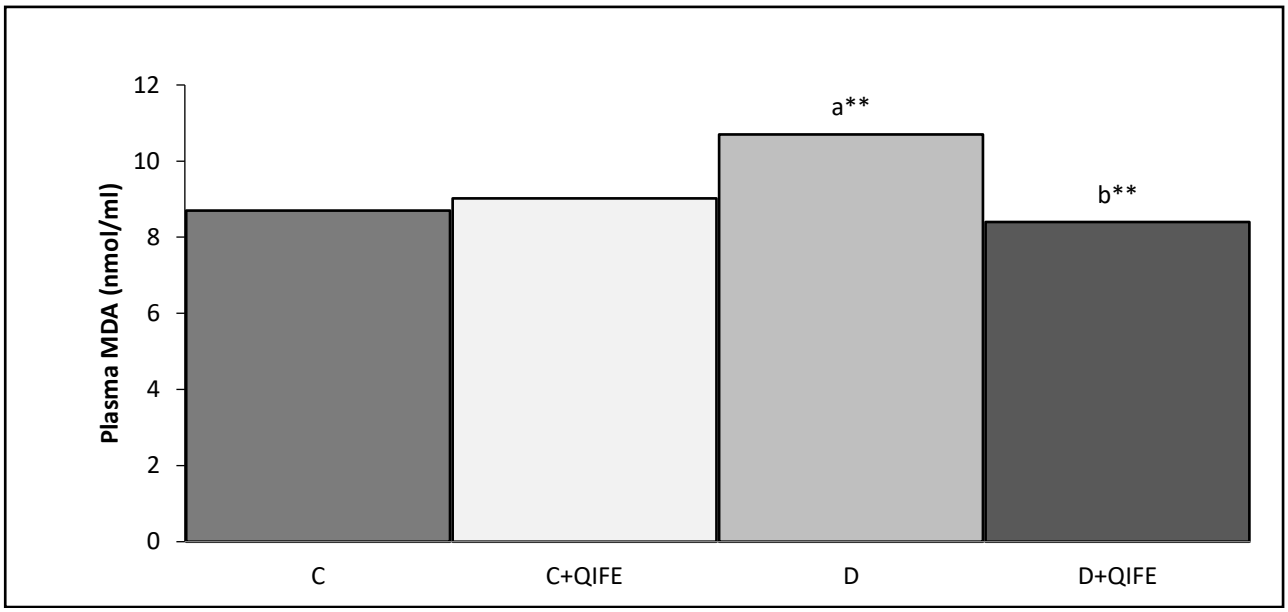

Figure 1. Plasma MDA (nmol/ml)

C: Normal control rats, C+QIFE: Control rats with orally administered *Q. ithaburensis* fruit extract, D: Streptozotocin-Nicotinamid induced diabetic rats, D+QIFE: Diabetic rats with orally administered *Q. ithaburensis* fruit extract. Values are expressed as mean ± SEM (standard error of the mean) for ten rats in each group, Statistical comparison: [a] C vs C+QIFE, [b] C vs D, [c] D vs D+QIFE, Statistical significance, ** $p < 0.01$

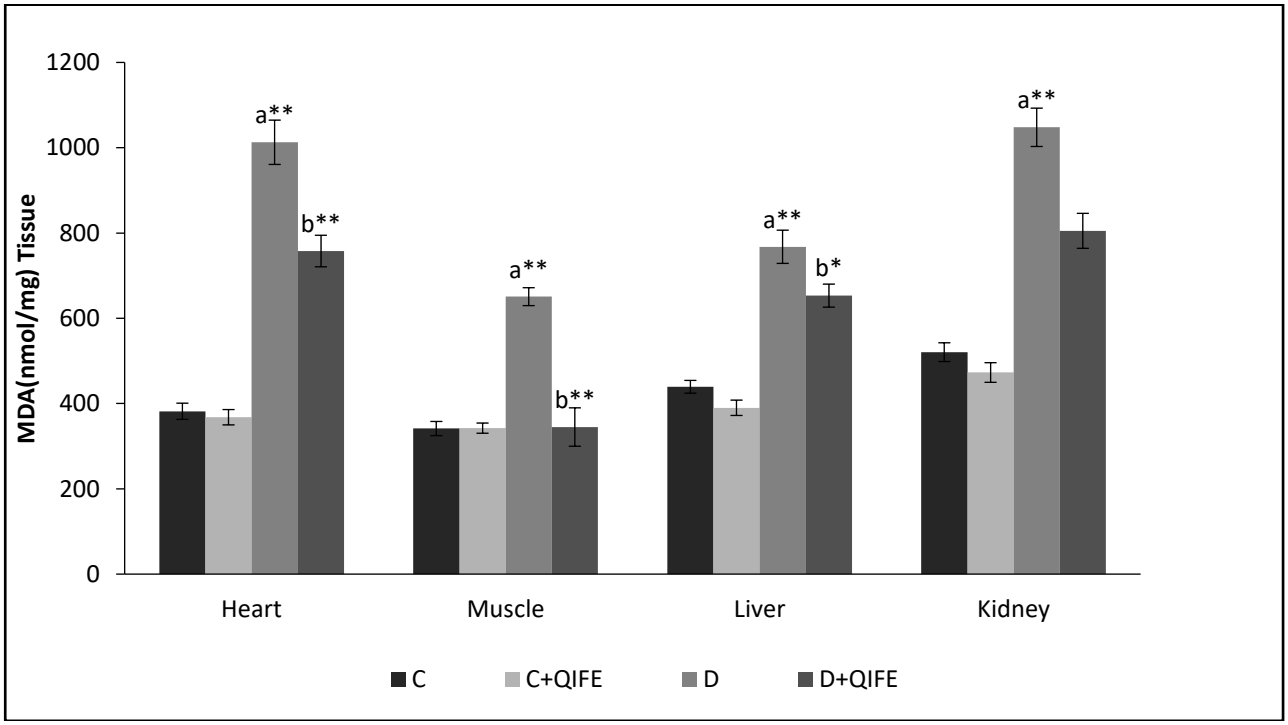

Figure 2. MDA(nmol/mg) Tissue

Values are expressed as mean ± SEM for ten rats in each group, Statistical comparison: [a] C vs C+QIFE [b] C vs D, [c] D vs D+QIFE, Statistical significance, * $p < 0.05$, ** $p < 0.01$, for abbreviations of study groups, see Figure 1.

4. Discussion and Conclusion

STZ-Nicotinamide administration is one of the commonly used methods for experimentally inducing type 2 diabetes in rats. While STZ selectively damages the insulin-secreting beta cells of the pancreas, nicotinamide (B3) partially protects the pancreatic β cells through nitric oxide-mediated mechanisms and causes moderate hyperglycemia in rats when administered together (Masiello et al.,1998; Szkudelski, 2012; Ghasemi et al., 2014). In this study, a moderate increase in fluid and food intake and a decrease in hyperglycemia, hyperlipidemia, and insulin levels in rats administered STZ-nicotinamide supported the findings of type 2 diabetes (Table 1). In addition, the increase in plasma and tissue MDA levels, SOD and GSH-Px antioxidant enzyme levels, and decrease

in PON and ARE enzyme levels in the diabetes group support research findings indicating elevated oxidative stress levels in diabetes (Sarandol et al., 2020; Bassalat et al., 2020; Tas et al., 2018; Tas et al 2024; de Gaetano et al., 2018).

Many plants are used for therapeutic purposes in diabetes and one of them is the *Quercus* species. It has been reported that phenolic acids (especially ellagic and gallic acids and their derivatives), flavonoids (especially flavan-3-ol), and tannins (Rakic et al., 2006; Taib et al., 2020; Makhlouf et al., 2019; Özcan, 2007) found in the structure of almost all *Quercus* species have therapeutic properties in diabetes (Doğan et al., 2015; Faten et al., 2022; Yin et al., 2018; Ema et al., 2020). In this study, we observed a significant decrease in serum glucose levels in the D+QIFE and C+QIFE groups, as well as a significant increase in insulin levels in the D+QIFE group. These effects may be attributed to the impact of flavonoids in *Q. ithaburensis* fruit extract on carbohydrate metabolism, when compared to the D and C groups, respectively. Because flavonoids have the ability to inhibit carbohydrate hydrolyzing enzymes such as α-amylase, α-glucosidase, and disaccharidase and/or affect insulin uptake by altering the GLUT4 mechanism (Bhandari et al., 2008; Jianwei et al., 2014; de Gaetano et al., 2018; Etxeberria et al., 2012; Anastasia et al., 2015). In addition, another reason for the decrease in blood glucose levels is the ability of flavonoids to regenerate the pancreas due to their strong antioxidant effects and this has been shown in many studies (Arora et al., 2021; Vinayagam & Xu, 2015; Matacchione et al., 2020; Attanayake et al., 2019). The increase in insulin levels in the D+QIFE group in this study confirms this. However, another important issue that we need to focus on in this study is that blood sugar levels were significantly lower in the C+QIFE group than in the control group. For this reason, QIFE extract may have a hypoglycemic effect on healthy individuals and its dose-related effects should be carefully evaluated and investigated. Also, we think that the decrease in TC levels in the C+QIFE and D+QIFE groups may be due to different action mechanisms of flavonoids in the QIFE extract such as reducing intestinal lipid absorption, bile acid chelation or inhibition of pancreatic lipase (Sun et al., 2020; Sugiyama et al., 2007; Gök et al., 2020). In addition, the hyperglycemia and hyperlipidemia we detected in the diabetes group in this study, and the changes in antioxidant enzyme levels may have contributed to the increase in plasma and tissue MDA levels in group D and these results are consistent with our previous studies (Taş et al., 2024, Taş et al., 2022; Taş et al., 2018; Bassalat et al., 2020). As it is known, one of the main targets of ROS is lipids and MDA is a product of lipid peroxidation and is one of the parameters widely used to indicate oxidative stress in the body (Tsikas, 2017; Wereski et al., 2022). Diabetes is a major risk factor for the development of atherosclerotic heart disease (Wereski et al., 2022; Katsiki, 2019; Shiyi et al.,2024). The decrease we observed in plasma and tissue MDA levels in the D+QIFE group in our study may be due to the direct antioxidant effect of the flavonoids found in *Q.ithaburensis* fruit extract as well as its antihyperglycemic and antihyperlipidemic effects as shown in this study. The importance of the antioxidant system in combating increased ROS in diabetes is very important, and antioxidant enzymes such as serum SOD and blood GSH-Px, CAT are very important in terms of their effects in preventing lipid peroxidation and the development of atherosclerosis (Chatuphonprasert et al., 2013; Sheweita et al., 2016). In this study, the increase in SOD and GSH-Px enzyme levels in the diabetic D group can be considered a response to increased oxidative stress. The increase in SOD and GSH-Px antioxidant enzyme levels in the C+QIFE group may result from the effect of *Q. ithaburensis* fruit extract on enzyme synthesis at the transcriptional level. The reason why there was no change in these enzyme levels in the D+QIFE group may have developed depending on the administration time or dose of this extract in the diabetic group.

Another antioxidant enzyme is PON 1; in our study, the decrease in PON and ARE levels in the diabetic group may be due to hyperglycemia, oxidative stress, and increased protein glycation in diabetes. It has been determined that paraoxonase binding to HDL is impaired due to changes in the regions where HDL binds to PON in diabetes (Abbott et al., 1995). When compared with this study, it is thought that the decrease in PON levels we found in our study may have developed due to this reason. PON1 is an HDL-dependent enzyme and exhibits atheroprotective properties by protecting both LDL and HDL from oxidation. In addition, ARE reflects the enzyme mass (Soran et al., 2015; Kotur-Stevuljević et al., 2020; Sun et al., 2017) and another factor that may contribute to decreased PON activity in diabetic conditions may be the decrease in ARE enzyme protein synthesis. The increase in both PON and ARE levels in the D+QIFE group that received *Q ithaburensis* fruit extract may be due to the antihyperglycemic and antihyperlipidemic properties of the QIFE extract as well as its ability to perform transcriptional enzyme synthesis as a powerful antioxidant.

Conclusion

We believe that *Quercus ithaburensis* subsp. *macrolepis* fruit extract exhibits antihyperglycemic, antihyperlipidemic, and hyperinsulinemic effects. Additionally, it significantly alleviates oxidative stress through its potent antioxidant activity as indicated by reduced levels of the lipid peroxide end product MDA (in plasma and tissues) and increased antioxidant enzyme activities. In addition, *Q. ithaburensis* fruit extract increased PON and ARE levels, which are very important in preventing the oxidation of lipoproteins, suggesting that it has a significant effect on the progression or prevention of micro-macrovascular complications in diabetes. However, another remarkable issue in this study is the significant decrease in blood sugar in the group receiving C+QIFE extract and the possibility that this may create the risk of hypoglycemia in healthy individuals. Therefore, when creating a treatment/support program, we recommend applying different dose and duration strategies well for healthy individuals and diabetic patients.

Thus, based on its strong antioxidant properties and ability to correct the impaired metabolism in diabetes, the fruit extract *Q. ithaburensis* can be considered a good phytotherapeutic agent to treat/support diabetes. However, further studies should be performed to fully understand its mechanisms of action.

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Conflict of interest: The authors declare that there is no conflict of interest.

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Research Article/Araştırma Makalesi

Investigation of Elemental Contents in Wild Goat Meat (*Capra aegagrus aegagrus*)

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Abstract: This study focuses on *Capra aegagrus aegagrus*, a subspecies of wild goat listed as vulnerable by the International Union for Conservation of Nature. Hunting of males aged 8 years and older is allowed due to their low reproductive capacity. This study aimed to analyze essential and potentially toxic elements in meat of male *C. aegagrus aegagrus* from a high altitude protected area in Mersin using inductively coupled plasma-optical emission spectroscopy (ICP-OES). Thigh muscle samples from 18 males aged 10 years and older were analyzed. Phosphorus, potassium, and calcium were the most abundant macro elements in the samples, while sodium and magnesium were the lowest. Iron was the most abundant microelement, followed in decreasing order by zinc, copper, manganese, boron, selenium, cobalt, chromium, vanadium, and nickel. Among the potentially toxic metals, lead had the highest concentration. Arsenic, antimony, strontium, cadmium, aluminum, and barium were found in lower concentrations. Tin was not detected in the samples. High levels of potassium, phosphorous, and iron suggest nutritional benefits but potentially toxic elements must be monitored to ensure safety.

Keywords: Element, metal, muscles, vulnerable, wild goat.

Yaban Keçisi Etindeki (*Capra aegagrus aegagrus*) Element İçeriğinin Araştırılması

Öz: Bu çalışma, Uluslararası Doğa Koruma Birliği tarafından hassas olarak listelenen yaban keçisi alt türü *Capra aegagrus aegagrus*'a odaklanmaktadır. Üreme kapasitelerinin düşük olması nedeniyle 8 yaş ve üzeri erkeklerin avlanmasına izin verilmektedir. Bu araştırma, Mersin'deki yüksek rakımlı bir koruma alanından alınan erkek *C. aegagrus aegagrus* etindeki temel ve potansiyel toksik elementleri indüktif eşleşmiş plazma-optik emisyon spektroskopisi (ICP-OES) kullanarak analiz etmeyi amaçlamıştır. Yaşları 10 ve daha büyük olan 18 erkekten alınan uyluk kası örnekleri incelenmiştir. Örneklerde fosfor, potasyum ve kalsiyum en fazla bulunan makro elementler olurken, sodyum ve magnezyum en düşük seviyedeydi. Demir en fazla bulunan mikro elementti ve onu azalan sırayla çinko, bakır, manganez, bor, selenyum, kobalt, krom, vanadyum ve nikel değerleri izliyordu. Potansiyel toksik metaller arasında en yüksek konsantrasyon kurşun olarak tespit edilmiştir. Arsenik, antimon, stronsiyum, kadmiyum, alüminyum ve baryum daha düşük konsantrasyonlarda bulunmuştur. Örneklerde kalay tespit edilememiştir. Yüksek potasyum, fosfor ve demir seviyeleri besinsel faydalara işaret etmektedir, ancak güvenliği sağlamak için potansiyel olarak toksik elementlerin izlenmesi gereklidir.

Anahtar kelimeler: Element, metal, kaslar, hassas, yaban keçisi.

1. Introduction

The wild goat (*Capra aegagrus*) is the ancestor of the domestic goat (*Capra hircus*), a species economically vital to millions worldwide (Taheri et al., 2023). There are five subspecies of *C. aegagrus* globally, with *C. aegagrus* ssp. *aegagrus* found in Turkey. The other subspecies are *C. aegagrus* ssp. *blythi, C. aegagrus* ssp. *chialtanensis*, *C. aegagrus* ssp. *cretica,* and *C. aegagrus* ssp. *turcmenica* (Shackleton, 1997). *C. aegagrus aegagrus* is distributed across Armenia, Azerbaijan, Georgia, Iran, Iraq, Russia, Afghanistan, Pakistan, and parts of eastern Asia, including Sindh and Baluchistan. This wild goat is listed as vulnerable (VU) in the World Red List published by the International Union for Conservation of Nature (IUCN) (Gundogdu and Ogurlu, 2009). Adult males measure 120-140 cm in length and weigh 50-85 kg, whereas females are 60-80 cm long and weigh 35-60 kg. Both sexes have beards but only males possess a distinctive black girdle from the shoulders to the forelegs, back, and neck. Both sexes have backward-curved horns, with males' horns measuring 100-145 cm and females' 25-30 cm. The age of the goats can be determined by the annual growth lines and ridges on their horns (Paşalı, 2014).

C. aegagrus aegagrus typically lives for 15-20 years. The mating season spans 3-4 weeks from mid-November to mid-December during which males engage in fights.

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These wild goats are herd animals, led by an old female. Males and females stay together from the breeding season until birthing (Gundogdu and Ogurlu, 2009). Outside of the breeding season, old and strong males live alone or in small groups of 2-3, while females and young males up to three years old form herds in the summer. Births usually occur in May after a gestation period of around five months (Ildoromi et al., 2019). Wild goats seek shelter in nooks and caves in rocky, steep areas at altitudes of 1500 meters or higher. They prefer densely wooded areas for safety and feed on tree and shrub shoots, various grasses, leaves, twigs, and wild fruits. Active during the day, they forage from early morning until dusk, resting at noon in the shade of rocks, dens, or between trees (Abbasian et al., 2004).

In Turkey, *C. aegagrus aegagrus* is included in hunting tourism, where hunting is permitted for males aged 8 years and older with reduced reproductive ability as determined by the competent authority. Hunting of females and males under 8 years is prohibited (Paşalı, 2014). Despite its inclusion in hunting regulations, little is known about wild goats, with existing studies focusing primarily on their behavior. Naturally fed wild goats are considered healthier and their meat is preferred for its superior taste. Red meat from naturally raised animals is a crucial source of animal protein, essential for human nutrition and health due to its vitamins, minerals, antioxidants, and various nutrients.

There is growing interest in the role of micronutrients (essential trace elements and vitamins) in optimizing health and preventing or treating diseases. Trace elements are vital in animal nutrition, required in minimal amounts for basic metabolic processes (Byrne and Murphy, 2022). Deficiencies in trace elements can cause losses comparable to those from infectious and parasitic diseases as these elements significantly influence disease resistance (Çamaş et al., 1994; Taghipour et al., 2021). Therefore, determining the concentration of elements in the muscles of wild goats, which inhabit rocky areas at altitudes of 1500 meters and higher and feed on grass, leaves, twigs, and wild fruits, will provide important nutritional information both for the animals and humans consuming them.

Additionally, animals serve as biomonitors of their environment; thus, the concentrations of toxic metals such as lead, cadmium, aluminum, and arsenic detected in these animals can indicate environmental contamination with these metals. These toxic metals have been reported to inhibit the absorption of other minerals and may indirectly cause mineral deficiencies (Gupta et al., 2021). Furthermore, as these animals are also consumed by humans, this poses an additional risk of human poisoning.

Assessing trace elements is thus crucial for determining nutrient deficiencies or toxicities within a population. There is currently insufficient information on the concentrations of essential elements and toxic metals in wild goats protected in the Cehennem Creek region of Turkey. The aim of this study is to investigate the concentrations of essential and toxic elements in the muscles of wild goats hunted as part of hunting tourism in Cehennem Creek, Mersin province and to use these goats as biomonitors for heavy metal environmental toxicity.

2. Material and Method

This study was conducted on the muscles of wild goats (*C. aegagrus aegagrus*) living in the Çamlıyayla-Cehennem Creek Wildlife Development Area in Mersin, Turkey and hunted as part of hunting tourism. The habitat of these wild goats is shown on the map in Figure 1. This area was granted special protection status by the competent authority in 2006, maintaining its natural structure away from settlements and industrial facilities. It is located at an altitude of at least 3000 meters above sea level. Permitted activities in the region include forestry, beekeeping, sheep and goat husbandry, ecotourism, and hunting tourism. Covering 27,610 hectares, this area is situated in the Mediterranean Region of southern Anatolia, east of the Central Taurus Mountains, at the foot of the Bolkar Range Mountains.

All studies were carried out as part of the Çamlıyayla-Cehennem Creek Wild Animals Capture, Monitoring, Inventory, and Management and Development Plan Construction Project initiated by the 7th Regional Directorate of the General Directorate of Nature Conservation and National Parks of the Ministry of Agriculture and Forestry (tender no: 2017/165207). The tradition of nomadic animal husbandry continues intensively in this area. Thigh muscle samples (approximately 50 g) were collected from 18 male wild goats aged 10 years and older, hunted during the 2018 and 2019 hunting seasons. Tissue samples were collected after the goats were hunted by hunters in the area. The samples were placed in separate glass jars, transported to the laboratory under a cold chain, stored at -21°C until analysis, and analyzed within one week.

The metals sought in thigh muscles were divided into 3 classes; (1) macro elements [calcium (Ca), phosphorous (P), potassium (K), magnesium (Mg), sodium (Na) (2) micro elements [boron (B), iron (Fe), zinc (Zn), manganese (Mn), cobalt (Co), chromium (Cr), copper (Cu), molybden (Mo), nickel (Ni), selenium (Se), vanadium (V)] (3) potentially toxic elements [aluminum (Al), arsenic (As), barium (Ba), cadmium (Cd), lead (Pb), antimony (Sb), tin (Sn) and strontium (Sr)] (World Health Organization, 1996).

Metal analyses were performed according to the United States Environmental Protection Agency (USEPA, 1998) method. Muscle samples were thawed at room temperature, minced thoroughly, and then dried. One gram of the dried, minced muscle was weighed directly into the clean Teflon digestion vessels at 1 mg sensitivity, homogenized with 8 mL of 65% HNO_3 and 2 mL of 30% hydrogen peroxide, and left for 20 minutes. The samples were then decomposed in a 1000-Watt microwave oven (MWS-2, Berghof Brand, Eningen, Germany) with medium pressure vessels using the program shown in Table 1.

Table 1. Microwave oven temperature program used for the decomposition of muscle samples

Step	Temperature (°C)	Power (W)	Time (mins)
1	200	70%	35
2	200	70%	20
3	100	40%	10

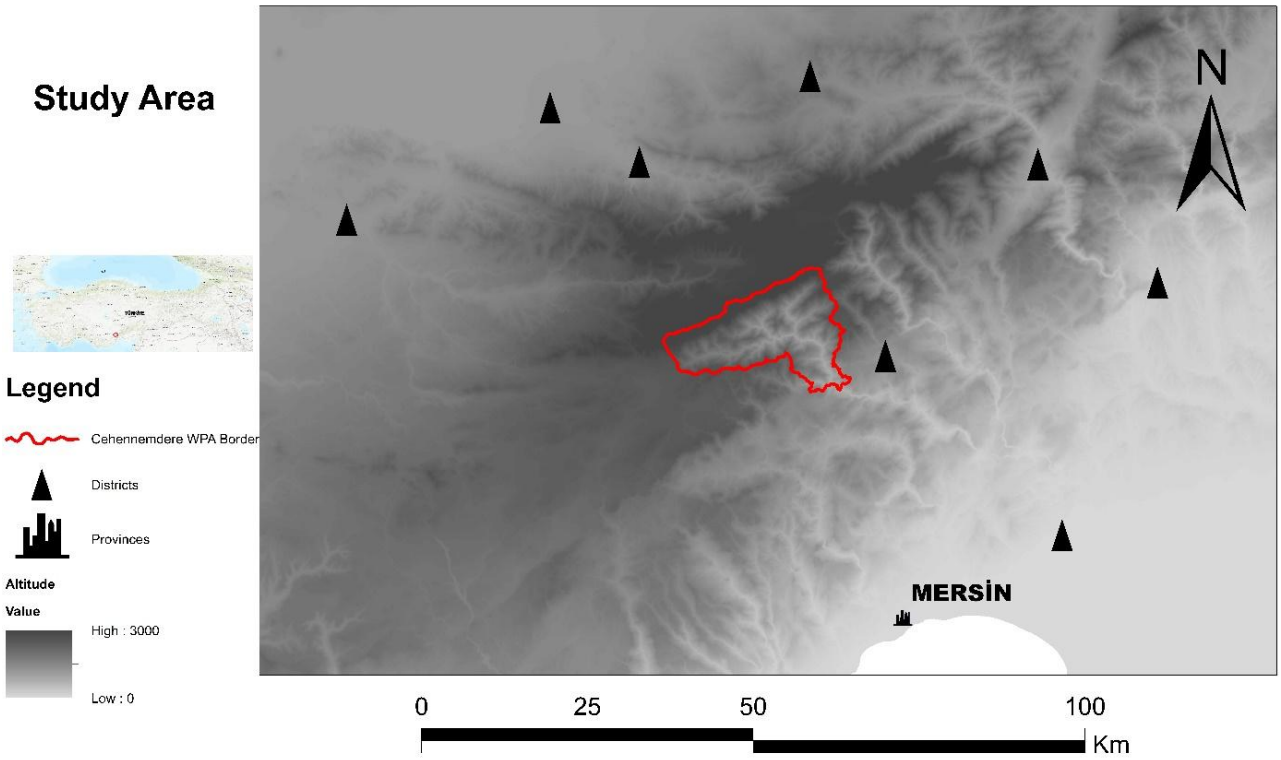

Figure 1. Habitat of sampled wild goats (*C. aegagrus aegagrus*)

All chemicals used in the analysis were of analytical or superior grade and fresh ASTM Type 1 grade ultrapure water was used throughout the study. Blank samples were used to account for the potential trace elements in the digestion chemicals. After digestion, vessels were cooled to room temperature, and digested samples filtered through Whatman No. 4 ashless filter paper and diluted to 15 mL with ultrapure water. Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) was then applied using the ICP-OES-Spectroblue series (SPECTRO Analytical Instruments GmbH, Kleve, Germany) (USEPA, 1998). The instrumental conditions for the ICP-OES measurements are summarized in Table 2. The method was calibrated using a 10-level calibration curve (concentration versus CPS) that was diluted with a 1% aqueous nitric acid solution from a primary multi-element standard (Multimix IV, Merck, Darmstadt, Germany). Quality control was checked against a certified reference material (Merck, Darmstadt, Germany).

Table 2. Inductively Coupled Plasma-Optical Emission Spectroscopy instrument conditions.

Parameters	Value
Plasma Power	1435 W
Pump Speed	30 rpm
Coolant Flow	13 L/min
Auxilary Flow	0.80 L/min
Nebulizer Flow	0.75 L/min
Number of replicates	3
Integration time (s)	3 s
Sample uptake rate (µL/min) (speed)	0.3 rps

The wavelengths used in ICP-OES, correlation coefficients, and calculated detection limits (DL) are provided in Table 3. All macro and micro element concentrations were expressed in mg/100 g dry matter (DM) and potentially toxic elements were expressed in µg/kg DM.

3. Results

The elements detected in the thigh muscles of wild goats and their minimum and maximum levels were given in Table 4. The highest macro elements found in the thigh muscles of wild goats were P, followed by K and Ca, with the lowest levels being Na and Mg (Table 4).

Microelement concentrations in wild goats were highest in Fe, followed in descending order by Zn, Cu, Mn, B, Se, Co, Mo, Cr and V, and lowest in Ni (Table 4).

4. Discussion

In a study (Dey et al., 2019) conducted with 16 Black Bengal goats without specifying age and sex, it was reported that Cu concentrations in the thigh muscle (0.84 mg/100 g) were quite close to the Cu concentrations found in our study, while Mn (0.86 mg/100 g) and Zn (9.09 mg/100 g) concentrations were quite high. However, the Mg concentration reported in the same study (0.051 mg/100 g) was considerably lower than the Mg concentration found in our study (81.32 mg/100 g). This may be explained by the fact that wild goats are more mobile than domestic goats. As it is known, Mg is an important element affecting muscle mass, muscle strength and performance (Castiglioni et al., 2024). On the other hand, Cu, Fe and Mn concentrations found in a study (Ivanović et al., 2016) in Serbian white goats and Balkan goats were considerably lower than the concentrations

found in our study. The same situation was found in the concentrations of Mn, Fe, Zn and Cu detected in the muscles of male goats bred as crossbred in Poland. Thus, it is understood that goats may show different mineral compositions according to their feeding habits and breed (Niedziółka et al., 2010).

Table 3. Wavelengths used in ICP-OES, correlation coefficients and calculated detection limit.

Element	Wavelenght (nm)	Correlation Coefficient (r^2)	Detection Limit (µg kg^{-1})
Aluminium	167.1	0.99978	0.06
Antimony	217.6	0.99982	1.80
Arsenic	189.0	0.99970	1.64
Barium	233.5	0.99990	0.03
Boron	249.7	0.99975	6.43
Cadmium	226.5	0.99987	0.01
Calcium	393.4	0.99845	2.14
Chromium	267.7	0.99954	0.08
Cobalt	228.6	0.99924	0.05
Copper	324.7	0.99966	0.63
Iron	238.2	0.99986	0.10
Lead	220.4	0.99973	0.16
Magnesium	285.2	0.99994	1.60
Manganese	257.6	0.99988	0.03
Molybdenum	202.1	0.99982	0.09
Sodium	589.6	0.99859	4.75
Nickel	221.6	0.99982	0.58
Phosphorous	213.6	0.99845	11.2
Potassium	766.5	0.99683	0.38
Selenium	204.1	0.99950	0.62
Strontium	421.6	0.99974	0.03
Tin	242.9	0.99983	0.80
Vanadium	309.3	0.99981	0.31
Zinc	202.6	0.99966	1.52

In contrast to wild goats, Alpine and Saanen kids exhibited the highest levels of K, followed by Na and P, with the lowest levels of Mg and Ca (Mioč et al., 2000). The influence of breed and sex on macro mineral concentration in muscles is minimal, although breed and sex significantly affect K and Mg levels (Park, 1990). Compared to wild goats, the muscles of young kids contain significantly lower levels of P and K, approximately equal levels of Mg and Na, and higher levels of Ca. Kid meat is noted for its high nutritional value due to its protein content, low fat content, and abundance of macro and microelements (Popov-Reljić et al., 1995). The high concentration of K in wild goat muscle tissue is noteworthy because K is essential for maintaining fluid balance, nerve function, and muscle contractions (Vaudin et al., 2022). Additionally, the Ca concentration suggests that wild goat meat could contribute to dietary Ca intake that is crucial for bone health (Tokysheva et al., 2022). The P level aligns with the requirement for ATP production and bone structure (Calvo and Lamberg-Allardt, 2015). The moderate Na content provides essential electrolyte balance without excessively contributing to the recommended Na intake limit (WHO, 2012). Mg is vital for enzyme activity and muscle function (Grober et al., 2015). Several factors, such as sex, age, cooking and processing methods, breed, and management systems, have been reported to influence Ca levels in goat meat. Goat meat contains more Ca than chicken and mutton and less P than beef. It has also been reported that cooked goat meat has much higher Na and Mg content than beef (Sheridan et al., 2003; Osman and Mahgoub, 2012).

Table 4. Element concentrations detected in wild goat thigh muscles (n:18).

Element	Concentrations	Minimum	Maximum
Macro elements (mg 100 g^{-1} Dry Matter)			
Calcium	587.91±65.9	518.41	724.67
Magnesium	81.32±7.9	67.89	99.50
Sodium	260.97±37.22	200.01	330.34
Phosphorous	1656.65±253.1	1298.69	2124.58
Potassium	1157.58±131.2	907.26	1399.83
Micro elements (mg 100 g^{-1} Dry Matter)			
Boron	0.35±0.05	0.25	0.46
Chromium	0.04±0.02	0.01	0.077
Cobalt	0.08±0.03	0.06	0.11
Copper	0.90±0.08	0.76	1.03
Iron	50.67±13.8	35.44	75.13
Manganese	0.50±0.01	0.20	0.69
Molybdenum	0.05±0.006	0.43	0.06
Nickel	0.02±0.01	0.01	0.06
Selenium	0.13±0.04	0.09	0.20
Vanadium	0.04±0.002	0.01	0.06
Zinc	4.10±0.6	2.96	5.1
Potentially toxic elements (µg kg^{-1} Dry Matter)			
Aluminium	4.97±1.2	1.21	17.14
Antimony	35.92±6.3	25.10	47.72
Arsenic	59.39±17.6	25.12	93.78
Barium	4.22±0.8	2.50	5.71
Cadmium	29.21±8.7	15.92	44.59
Lead	654.67±157.3	298.98	898.47
Strontium	29.41±8.8	2.99	45.47
Tin	<DL	-	-

In a study conducted in Alpine and Saanen kids, it was reported that the highest level of Zn was found in the muscles, followed by Cu and Fe and the lowest level was Mn (Mioč et al. (2000). Thus, it is seen that breed and age have a significant effect on micronutrient concentrations in muscles of goats.

Fe is an element particularly associated with anemia. Goat meat can be a good source of heme iron, which has higher bioavailability compared to non-heme iron from plant sources (Park and Attaie, 1988). Zn concentration in wild goat muscle is important because zinc is vital for immune function and cellular metabolism (Prasad, 2013). Zn concentration in wild goats has been shown to be higher than in beef (Hoffman et al., 2003). Cu is an essential trace element for mammals. Besides its role in Fe metabolism, the need for Cu stems from its role in numerous biological processes, including antioxidant

defense, neuropeptide synthesis, and immune function (Bost et al., 2016). Co level is essential for vitamin B12 synthesis (Spataru, 2024). The Fe level in cooked goat meat is almost the same as in cooked beef but about twice as high as in chicken meat (Osman and Mahgoub, 2012).

In a study conducted in Omani and Somali goats, V level in muscles was found to be 0.003 and 0.03 mg/100 mg DM, Cr level was found to be 0.01 and 0.11 mg/100 g, and Mo level was found to be 0.13 and 0.16 mg/100 mg DM, respectively (Osman and Mahgoub, 2012). V level in wild goats was close to Somali goats, Cr level was higher than Omani goats but lower than Somali goats and Mo level was lower than both goats. This situation can be explained by race, age, and nutritional status.

According to our knowledge, there is no study on B in goat meat. However, boron is considered as an essential mineral for humans and animals by the World Health Organization (World Health Organization, 1998). In a study, it was found that 70 mg/kg B was added to the feed and fed to male goats for 6 months without affecting growth but there was a significant increase in serum Mg concentrations of male goats fed with B at 24th week (Ibrahim et al., 2023).

Although V is widely used in industrial plants, no harmful effects have been observed in animals or humans. As an analogue of P, it interferes with P metabolism, mimics growth factors, and is involved in cell proliferation, repair and angiogenesis. It is also used in the treatment of diabetes as it has insulin-like effects. The concentration of V in soil and plants, including vegetables, is known to increase near industrial activities (Altaf et al., 2021). However, since there are no industrial facilities near wild goats, it was thought that this could come from other sources.

It has been reported that Cr in goat diets has no effect on carcass characteristics of goats but may improve meat quality with higher protein content, leaner and healthier fatty acids for human consumption (Lalhriatpuii et al., 2024). Cr is known as glucose tolerance factor. It is known to potentiate insulin activity by stimulating insulin receptors on the cell membrane, thereby enabling cells to take up glucose, which maintains blood glucose levels (Zhao et al., 2022). In this study, Cr concentrations in muscles were very close to the Cr concentrations found in Bengal goats by Lalhriatpuii et al (2024). This indicates that there is no difference in Cr concentrations in muscles between wild goats and domestic goats.

The mean Mo concentration detected in wild goat meat (0.05 mg/100 mg DM) was lower than the mean Mo concentration in red deer muscle (0.42 ± 0.07 mg/100 g wet weight) (Skibniewski et al, 2015). Since the Mo concentration measured in this study was lower than those observed in other free-living ruminant species and lower than those found in farmed ruminants, it should be stated that wild goat meat is not a good source of Mo for human dietary requirements.

When evaluated in terms of potentially toxic metals, it is seen that Pb is the highest in goat muscles followed by As, Sb, Sr, Cd, Al, and Ba in decreasing order and Sn metal is not found.

Among the potentially toxic elements, Pb levels (654.67 ± 157.3 ppb) raise concerns given that chronic exposure to lead is associated with neurological and developmental disorders (Bjørklund et al., 2024; Shibeeb et al., 2024). The As levels (59.39 ± 17.64 ppb) also warrant attention, as arsenic exposure can contribute to various health issues like skin lesions and cancer (Tchounwou et al., 2023). Despite the presence of these elements, the concentrations of Sn were below the detection limit and Cd (29.21 ± 8.7 ppb) remains within acceptable limits (European Food Safety Authority., 2012). In the Turkish Food Codex Contaminants Regulation, MRL values in goat meat are given as 200 µg/kg for Pb and 50 µg/kg for Cd. No MRLs were set for other metals in goat meat. Thus, based on the lead concentration detected in wild goat meat according to the legislation in Turkey, it should be stated that health problems may be encountered in people consuming this type of meat.

The levels of Sr (29.41±8.8 µg/kg) observed in goat thigh muscle are noteworthy as strontium is not typically associated with adverse health effects at these concentrations (Curtis et al., 2021). Conversely, the presence of Ba (4.22±0.8 µg/kg) and Al (4.97± 1.4 ppb) at elevated levels suggests potential environmental contamination sources that could affect muscle mineral composition (Pi et al., 2019). Monitoring these levels in wild goat meat is crucial to ensure they remain within safe consumption limits and align with food safety standards set by health organizations like the EFSA and WHO.

Comparing the elemental composition of wild goat muscle tissue to other meats like beef, pork, and chicken, goat meat often has lower fat and cholesterol content while being rich in essential nutrients (Suman and Joseph, 2010). Its high Fe and Zn content makes it a good option for individuals requiring higher intake of these minerals. However, regular monitoring of potentially toxic elements is crucial to ensure safety, particularly in regions with high industrial activity or environmental pollution that could increase contamination (Korish et al., 2020).

5. Conclusion

The analysis of elemental concentrations in wild goat muscle tissue offers valuable insights into the nutritional composition of wild goat meat and its potential health implications. The data obtained can be contextualized within nutritional guidelines and compared to other commonly consumed meats. Additionally, assessing the levels of potentially toxic elements ensures the meat's safety for consumption. In summary, wild goat muscle tissue is a rich source of macro and microelements essential for human health. The high levels of K, P, and Fe make it particularly beneficial. However, it is crucial to monitor the concentrations of potentially toxic elements to ensure they remain within safe consumption limits. Further research comparing the elemental profiles of different cuts and cooking methods would provide additional valuable insights into optimizing the nutritional benefits of goat meat.

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A New Type Covering Setae Morphology in Crab Spiders (Araneae)

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Abstract: The present study aims to determine the covering setae in two crab spiders using scanning electron microscopy (SEM). The setae on the prosoma of *Runcinia grammica* (C. L. Koch, 1837) and *Thomisus onustus* Walckenaer, 1805 were examined. This study reveals a new type of covering setae in the members of Thomisidae family.

Keywords: Cuticular structure, SEM, setae, thomisidae, araneae, surface morphology.

Yengeç Örümceklerinde (Araneae) Yeni Bir Tip Örtü Setası Morfolojisi

Öz: Mevcut çalışmada taramalı elektron mikroskobu kullanılarak iki yengeç örümceği türünde örtü setalarının morfolojisinin belirlenmesi amaçlanmıştır. *Runcinia grammica* (C. L. Koch, 1837) ve *Thomisus onustus* Walckenaer, 1805 türlerinin prosomaları üzerindeki setalar bu amaçla incelenmiştir. Thomisidae familyası üyelerinde ilk kez görülen bir örtü setası tipine rastlanılmıştır.

Anahtar kelimeler: Kütikular yapı, sem, seta, thomisidae, araneae, yüzey morfolojisi.

Setae in spiders have different morphologies and diverse functions depending on their location on the body (Ovtsharenko 1985; 1989). Only 13 of the 134 known spider families have been studied on setae morphology (Townsend & Felgenhauer, 1998). Although the studies have mostly focused on ground spiders (Gnaphosidae), lynx spiders (Oxyopidae), and jumping spiders (Salticidae), there have been little or no studies on other families.

Covering setae are specialized, hair-like structures found on the bodies of spiders. These are not just ordinary hairs; they are essential for a spider's survival, locomotion, and environmental adaptation. They are located mostly on the abdomen and may also cover the cephalothorax, legs, pedipalps, and spinnerets. They have no connection with sensory receptor cells (Townsend & Felgenhauer, 1998). It has been recognized that there are 10 different types of setae on the cuticle of all spiders. The morphology of covering setae varies among different spider genera. Researchers have identified several major types of covering setae such as plumose, squamose, lanceolate, pinnate, arborate, and sicate forms, each with distinct characteristics (Zakharov & Ovtsharenko, 2015).

In the Thomisidae family, covering setae were studied in *Stephanopis* cf. *scabra* L. Koch, 1874 and *S. cambridgei* Thorell, 1870 and found 3 different types of setae that are finger-shaped with dentation. The dentate structure of the setae is related to the fact that they live among the debris and use these debris as camouflage (Gawryszewski, 2014).

The aim of this study is to determine the new covering setae type morphology from the species *Runcinia grammica* (C. L. Koch, 1837) and *Thomisus onustus* Walckenaer, 1805 that live on vegetation and belong to the Thomisidae in the Araneae class.

The species of the Thomisidae family used in this work were obtained from the Niğde Ömer Halisdemir University Arachnology Museum (NOHUAM). These species are *Runcinia grammica* (C. L. Koch, 1837) (♀) and *Thomisus onustus* Walckenaer, 1805 (♀♂). The scanning electron microscope (SEM) was used in the Central Research Laboratory of Niğde Ömer Halisdemir University to determine the seta morphology of these crap spiders. For each species, these body parts (prosoma, opisthosoma, and legs) were placed on the stapes in the proper position and the surface of these specimens was coated with gold with a Sputter Coater (Cressingto Auto 108) brand device to obtain clearer images. The specimens were then photographed using an EVO LS 10 ZEISS device to examine the surface morphology (Fig. 1).

The setae morphology on the prosoma of *Runcinia grammica* (C. L. Koch, 1837) and *Thomisus onustus* Walckenaer, 1805 from Thomisidae was examined by SEM and a new covering type setae morphology has been identified for the first time. (Figs. 2-4).

Studies on the setae of spiders have mostly focused on members of the families Salticidae, Oxyopidae, and Gnaphosidae. Considering the spider families examined, lanceolate, spatulate, and plumose setae are commonly observed in spiders. There are almost no studies covering setae morphology in members of the family Thomisidae (Townsend & Felgenhauer, 1998; Gawryszewski, 2014; Baltayeva et al., 2024). According to these studies, Baltayeva reported that there are no cover setae in species *Synema plorator* (O. Pickard-Cambridge, 1872), *Xysticus laetus* Thorell, 1875, and *Diaea dorsata* (Fabricius, 1777), while Gawryszewski reported that there are 3 different types of finger-shaped setae with denticles in species of

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genus *Stephanopis*. He also reported that crab spiders *Stephanopis* spp. adapt to their environment by attaching debris to their bodies with the help of setae. In this study, finger-shaped setae morphology was found in *Runcinia grammica* (C. L. Koch, 1837) and *Thomisus onustus* Walckenaer, 1805 from Thomisidae family. This type of setae is similar to that of the crab spider genus *Stephanopis* but of a different type. While the other types (type I, II, and III setae) have denticles, this new type of seta does not have denticles.

Figure 1. SEM imaging process

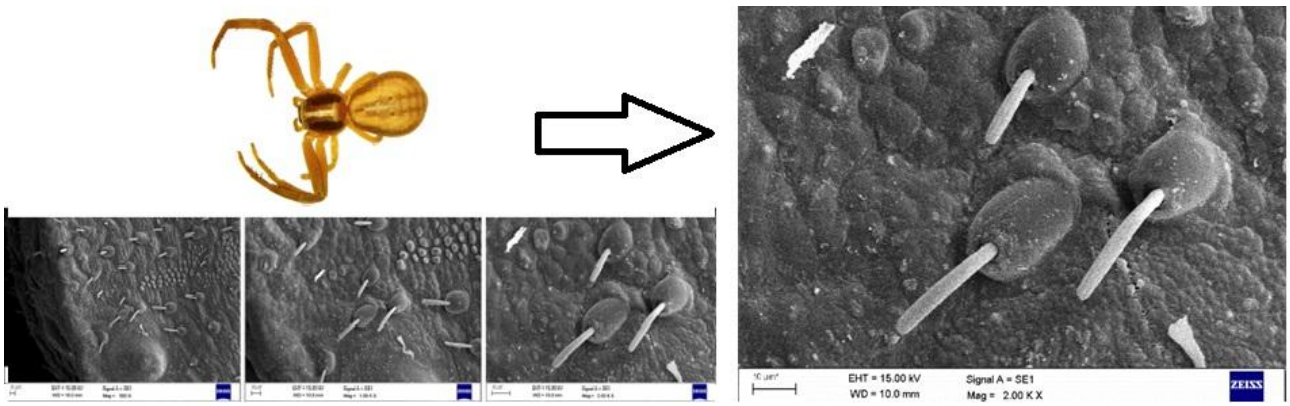

Figure 2. Digital (habitus) and SEM (covering setae) photos of *Runcinia grammica* (C. L. Koch, 1837)

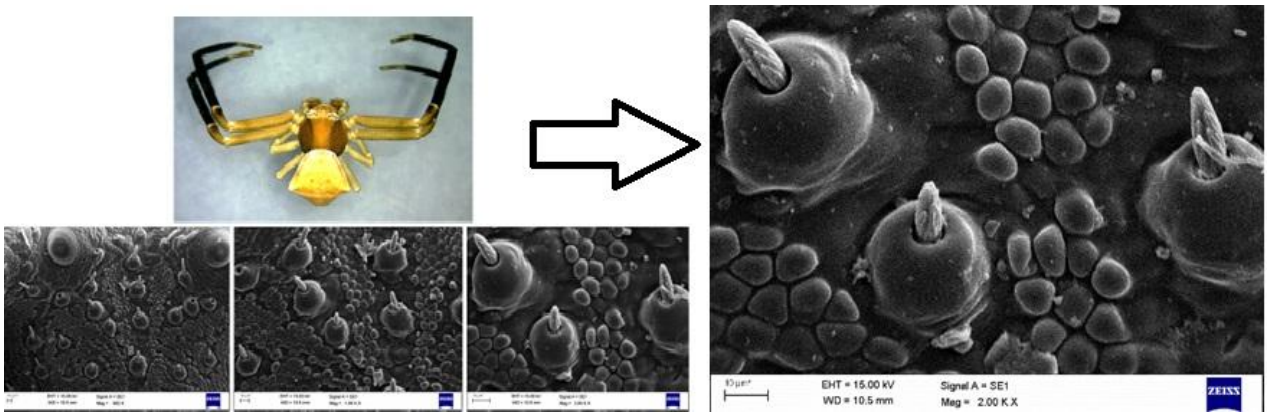

Figure 3. Digital (habitus) and SEM (covering setae) photos of male of *Thomisus onustus* Walckenaer, 1805.

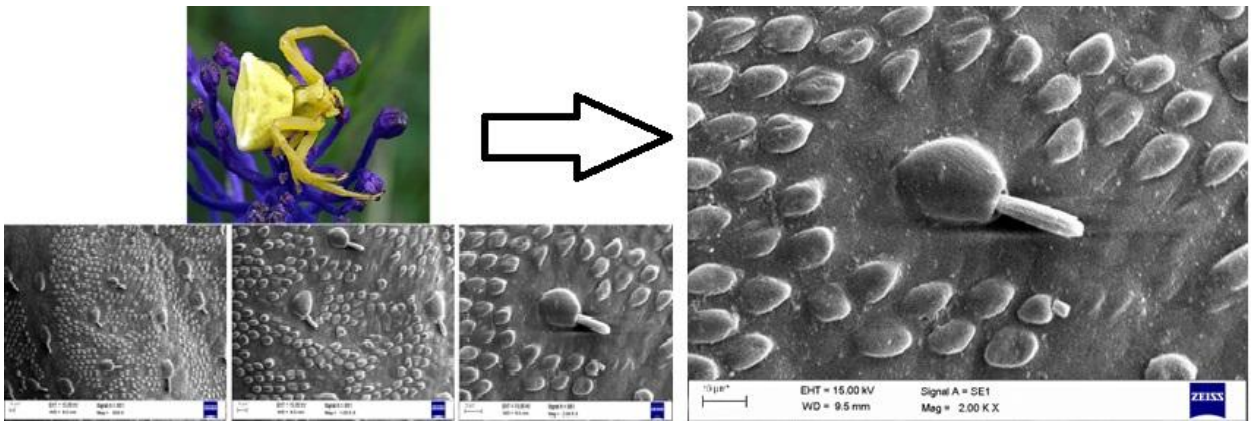

Figure 4. Digital (habitus) and SEM (covering setae) photos of female of *Thomisus onustus* Walckenaer, 1805.

Comparing the new type setae morphology of *Runcinia grammica* (C. L. Koch, 1837) and *Thomisus onustus* Walckenaer, 1805, it was observed that the setae length was longer in *R. grammica* (C. L. Koch, 1837). In addition, it

was observed that in *Thomisus onustus*, this setae type morphology is different in length for both sexes, with the female having a slightly longer setae length than the male. More studies should be conducted to learn about the covering setae in the crab spider species belonging to the Thomisidae family.

Ethics committee approval: Ethics committee approval is not required for this study.

Conflict of interest: The authors declare that there is no conflict of interest.

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A Review of the Status of Pygmy Cormorant *Microcarbo pygmaeus* in Cyprus Following the First Record in 25 Years

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Abstract: This study reports on the status of Pygmy Cormorant in Cyprus, following the first observation of the species since 1999. Notably, this sighting represents the second record of the species from the northern part of the island. The individual was documented at the Agia Eirini reservoir (Akdeniz), a wetland situated between the Kyrenia and Nicosia districts in Northern Cyprus. The same species was observed again at the same location two months after the initial sighting, raising two potential hypotheses: the establishment of the same individual in the area or the arrival of a different specimen which would suggest a tentative expansion of the species' range.

Keywords: Pygmy Cormorant, North Cyprus, first record, vagrant, range expansion.

Küçük Karabatak (*Microcarbo pygmaeus*)'ın 25 Yıl Sonra Kıbrıs'tan İlk Kaydı ve Durumunun Değerlendirilmesi

Öz: Bu çalışma, 1999 yılından bu yana ilk kez gözlemlenen Küçük Karabatak'ın Kıbrıs'taki durumu hakkında rapor sunmaktadır. Bu gözlemin, türün adanın kuzey kesiminden ikinci kaydı olması açısından önemlidir. Birey, Kuzey Kıbrıs'ın Girne ve Lefkoşa ilçeleri arasında yer alan Agia Eirini sulak alanında (Akdeniz) gözlemlendi. Aynı tür, ilk gözlemden iki ay sonra aynı yerde tekrar gözlemlendi ve bu durum iki olası hipotezi gündeme getirdi: Aynı bireyin bölgede yerleşmesi veya türün yayılım alanının geçici olarak genişlediğini düşündürecek farklı bir örneğin gelmesi.

Anahtar kelimeler: Küçük Karabatak, Kuzey Kıbrıs, ilk kayıt, nadir, dağılış genişletme.

The Pygmy Cormorant *Microcarbo pygmaeus* is a member of the Phalacrocoracidae family of seabirds listed as Least Concern by the IUCN with an increasing population trend (BirdLife International, 2019). The species' distribution range extends from south-eastern Europe to south-western Asia with the highest breeding densities concentrated in the Danube Delta and Black Sea (Schogolev *et al*., 2005; BirdLife International, 2024).

In Cyprus, the species is considered as a vagrant or formerly scarce and irregular passage migrant and winter visitor with the nearest resident population in Turkey from where most individuals are presumed to have migrated (Flint & Richardson, 2024). The first record on the island dates from late August 1982, with two individuals found at Phasouri/Akrotiri reed-beds (Flint & Stewart, 1992). Since then, there have been just over 20 records, comprising a total of 38 different birds (Colin Richardson pers comm). The most recent record was of two individuals at Evretou Dam on 2 September 1999. The onset of these occurrences aligns with a significant increase in the construction of fish-stocked dams, where the vast majority of sightings have been observed (Flint & Richardson, 2024).

The species' breeding population suffered a dramatic decline during the 1950s and in the following decades (Ławicki *et al*., 2012). This reduction was particularly pronounced in Turkey during the 1990s, coinciding with the last recorded observation in Cyprus, after which there was a prolonged absence of sightings.

On 16 April 2024, a team from the "Cyprus Wildlife Research Institute (CWRI)", established under the NGO Taşkent Nature Park, identified an individual of Pygmy Cormorant during their monthly census at the Agia Eirini (Akdeniz) reservoir (35°15'25"N, 32°59'18"E). This site is an artificial wetland located between the districts of Kyrenia and Nicosia in the western part of Northern Cyprus (Fig. 1) and it is included in the list of regularly visited areas as part of the Monitoring Project for Wetland Birds, developed by CWRI, which spans Northern Cyprus.

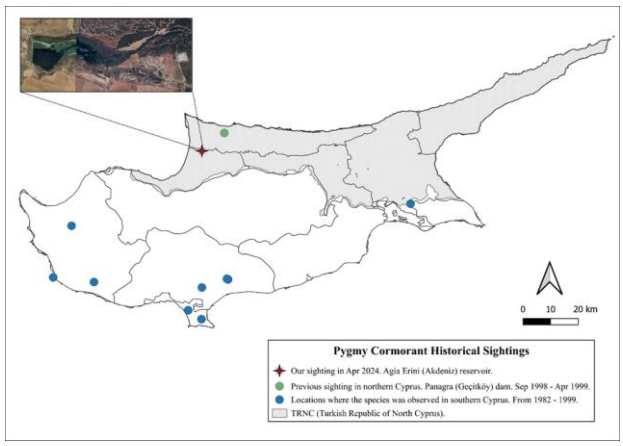

Figure 1. Locations where the Pygmy Cormorant has been observed in Cyprus and the satellite view of Agia Eirini (Akdeniz) reservoir.

This observation was made at 10:35 a.m. under favorable weather conditions (calm, clear, and 27°C). It lasted approximately 45 minutes, ending at 12:15 p.m. During this time, the individual remained perched on a branch, turning and grooming its plumage, until it

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eventually took flight and disappeared into the reedbeds (Fig. 2). Based on its plumage, it was most likely a juvenile as in comparison with an adult, a more brownish plumage can be observed in general with a whitish chin and a duller, paler belly (Orta *et al*.,2020). After this encounter, the team and other birdwatchers revisited the site on consecutive days but were unable to find any further trace of the species.

Figure 2. Photographs from the Pygmy Cormorant's first sighting. Image a) Individual perched on a branch. Image b) Individual in-flight crossing the reservoir.

Two months later, on 9 July 2024 a Pygmy Cormorant was found again at the same reservoir at 07:15 a.m. The observation lasted two and a half hours until the census was completed at 9:40 a.m. The bird took several short flights and appeared comfortable in its environment displaying diving and swimming behavior for quite some time.

During this observation, the plumage characteristics differed from those noted in April. Various features were evident, some of them characteristic of juveniles and others more typical of adults. The wing coverts displayed numerous small white dots along the feather edges, possibly indicative of retained juvenile plumage. Additionally, moulting and the development of adult flight feathers were apparent. The overall body coloration appeared more brownish, resembling that of a juvenile bird, though the throat and breast were not as light as observed in April (Fig. 3). These characteristics suggest that the individual may be in a transitional stage between juvenile and adult that is typical for this species until reaching sexual maturity at 3 to 4 years of age (BirdLife International, 2019).

On 11, 12, and 23 July, 8 August, and 4 September 2024, the same individual was spotted again exhibiting similar behaviors to those noted during previous sightings.

This new record of Pygmy Cormorant on the island, after a remarkable absence of 25 years, represents the second historical sighting of this species in Northern Cyprus and its first photographic documentation. The fact that this exceptionally rare species was observed in Cyprus on two separate occasions, two months apart, suggests two plausible hypotheses.

Figure 3. In-flight image of the Pygmy Cormorant spotted in July. Note that several primary feathers are missing and a few white spots on the shoulders, indicating the individual is undergoing a summer moult.

The absence of observation during May-Jun can be explained, firstly, by either a lack of ornithological activity in the area or the bird's concealment among the dense vegetation during census days. During this period, the bird may have been undergoing a moulting process, possibly being in an intermediate state (as shown in Fig. 3). Alternatively, it is possible that two different individuals were observed during this time. This scenario would imply two new different sightings of this species occurred within a two-month period—a relatively short time frame for such a rare species—and within the same vicinity.

In either case, this is of great interest as it may indicate a tentative expansion of the species' range and potentially lead to an increase in future sightings in Cyprus as seen in the 1990s (Flint & Richardson, 2024). This possibility could be related to the findings of Ławicki *et al*. (2012) that suggest a recovery and increase in the breeding population in southern and south-eastern Europe. Additionally, this trend may be supported by observations in nearby regions such as Egypt which had its first historical sighting in 2022, the Jordan Valley with its first sighting in 20 years in 2018, and Kuwait which recorded its 3rd historical sighting in 2013, among others (Blair *et al*., 2024).

These observations are truly significant and highlight the importance of continued monitoring efforts. Whether they reflect a single bird's behavior or indicate a broader trend, ongoing research is crucial for understanding the species' presence and status in Cyprus.

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