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RESEARCH ARTICLE

Research into the Presence of Shiga Toxin-producing Escherichia coli in Human and Cattle Feces with Culture, ELISA and Molecular Methods

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ABSTRACT

According to 2021 European Food Safety Authority (EFSA) data, Shiga-toxin (stx) producing *Escherichia coli* (STEC) is the fourth most frequently observed zoonotic agent in humans after *Campylobacter, Salmonella* and *Yersinia*. It may cause very serious infections like hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). In 2011 it caused a large epidemic, leading to the death of 54 people and the development of HUS in several people in Germany. For diagnosis, the detection of STEC bacteria is an important marker to indicate the formation of the toxin. In this study, the aim was to determine the frequency of STEC in patients referred for fecal cultures and to identify the colonization rates of this microorganism among cattle in an integrated meat facility. A total of 250 human fecal samples and fecal samples from 180 cattle collected from an integrated meat facility were investigated for the presence of STEC. None of the samples from humans had STEC identified. Of the animal samples, 11 were positive with enzyme linked immunosorbent assay (ELISA). Of the samples positive with ELISA, 8 were also positive with polymerase chain reaction (PCR). Of the samples positive with PCR, 3 could proliferate on CROMagar STEC medium. Of the 3 isolated bacteria, 1 was serotyped as O103:NM and the other two could not serotyped. The majority of studies performed for the detection of STEC in our country provide information about the O157 serotype; however, it is necessary to identify all strains producing stx with the multiplex PCR method as non-O157 strains may be responsible for large epidemics.

Key words: ELISA, Feces, PCR, Shiga toxin-producing *Escherichia coli*

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İnsan ve Sığır Dışkı Örneklerinde Shiga Toksin Üreten Escherichia coli Varlığının Kültür, ELİSA ve Moleküler Yöntemlerle Araştırılması

ÖZ

Shiga Toksin üreten *Escherichia coli* (STEC) 2021 EFSA verilerine göre insanlarda *Campylobacter, Salmonella* ve *Yersinia* türlerinden sonra en sıklıkla örülen dördüncü zoonotik etkendir. Hemolitik üremik sendrom (HÜS), hemorojik kolit (HK) gibi çok ciddi komplikasyonlara neden olabilmektedir. 2011 yılında Almanya'da 54 kişinin ölümüne ve birçok kişide HÜS oluşumuna sebep olan salgın gibi büyük salgınlara neden olabilir. Tanıda STEC bakterisinin tespiti ve toksin oluşumunu göstermek önemli bir yol göstericidir. Bu çalışmada, dışkı kültürü istemi yapılan hastalarda STEC sıklığının belirlenmesi ve bir entegre et tesisinde bulunan sığırlarda bu mikroorganizmanın kolonizasyon oranlarının saptanması amaçlanmıştır. İnsanlardan toplam 250 dışkı örneği ve bir entegre et tesisinden toplanan 180 sığıra ait dışkı örnekleri STEC varlığı yönünden incelenmiştir. Örnekler, CHROMagar STEC besiyeri ve MacConkey sıvı besiyerlerinde ekimi yapılmış, daha sonra enzyme linked immunosorbent assay (ELISA) ve polimeraz zincir reaksiyonu (PZR) yöntemleri kullanılarak STEC varlığı araştırılmıştır, PZR'de pozitif çıkan patojen gen bölgeleri için dizileme, kültürde üreyen STEC'ler için serotiplendirme yapılmıştır. İnsanlarda araştırılan örneklerin hiçbirinde STEC tespit edilmemiştir. Hayvan öreklerinin 11'i ELISA ile pozitif bulunmuştur. ELISA yöntemiyle pozitif bulunan örneklerin 8'i aynı zamanda PZR yöntemiyle de pozitif saptanmıştır. PZR yöntemiyle pozitif bulunan örneklerin 3'ü CROMagar STEC besiyerinde üretilebilmiştir. İzole edilen 3 bakteriden 1'i O103:NM olarak serotiplendirilmiş diğerleri serotiplendirilememiştir. Ülkemizde STEC varlığının tespiti için yapılan çalışmaların büyük bir kısmı O 157 serotipi hakkında bilgi vermektedir, ancak O 157 dışı suşlarında büyük salgınlara sebep olabilmesi nedeniyle multipleks PZR yöntemiyle, Stx üreten tüm suşların tespit edilmesi gerekmektedir.

Anahtar kelimeler: Dışkı, ELISA, PCR, Shiga Toksin üreten *Escherichia coli*

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INTRODUCTION

Escherichia coli (E. coli) is generally a harmless commensal bacteria. However, some E. coli strains may cause disease if they invade intestinal mucosa, release toxins or enter blood circulation. These strains are pathogenic E. coli with the ability to cause infection (Torres2010). Shiga toxin (Stx) producing E. coli (STEC) is among these pathogens and may cause very serious complications like hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans (Paton and Paton 1998). Stx is one of the most important virulence factors in pathogenesis. Stx comprises two major groups called Stx1 and Stx2 and these groups also contain several subgroups (Bergan et al. 2012). Not all Stx variants cause serious disease; only some are associated with serious disease. For example, while HC and HUS are mostly associated with Stx1a, the strains Stx1c and Stx1d are less associated with infections in humans. In the Stx2 group; Stx2a, Stx2c, Stx2d, Stx2b and Stx2e were found to be more virulent than Stx2f and Stx2g (Harada et al. 2023). STEC strains firstly colonize intestinal mucosa, then form a characteristic histopathological lesion. Formation of this lesion, called an attaching and effacing (A/E) lesion, is controlled by the locus of enterocyte effacement (LEE) pathogenicity island. The Eae gene region codes intimin and is a component of the LEE gene region (Garmendia et al. 2005; Torres2010; Prager et al. 2011). STEC secretes alpha hemolysin (hly) and this enzyme forms a cavity in the cell membrane and degrades erythrocytes. There are four genes responsible for the synthesis of alpha hemolysis; these are HlyA, HlyB, HlyC, and HlyD (WelchandPellett 1988; Welch1991; Eklund2005). For STEC infections, cattle are the most important reservoir (Torres2010). In this study, an attempt was made to detect the presence of STEC with culture, micro ELISA and multiplex classic polymerase chain reaction (PCR) methods in cattle feces and selected human feces.

MATERIALS and METHODS

Collection of Samples

A total of 250 Human fecal samples were investigated macroscopically and microscopically from July 2014 to December 2014. Samples comprised with runny/soft texture and/or containing leukocytes, erythrocytes or leukocytes and erythrocytes, watery and mucous-rich features.

Animal feces were obtained from a meat and meat products integrated facility under observation of a veterinarian. A total of 180 samples were taken, with 90 obtained in July 2014 and 90 obtained in August 2014, from the anal region of cattle using single-use clean and dry rods.

Culture

A loop of feces was taken from the collected material and firstly inoculated on CROMagar STEC medium. Then a loop of feces was inoculated to MacConkey broth were incubated at 37 °C for 16-24 hours and then examined for whether mahogany-colored colonies formed on CROMagar STEC medium. Colonies with blue color, transparent or with other colors were not accepted as STEC. *E. coli* was confirmed by definition with the Vitek 2 Compact (Biomerieux, France).

ELISA

The presence of stx1 and stx2 toxins were investigated in MacConkey broth by ELISA method, and also Samples positive for the presence of Stx with the ELISA method on MacConkey broth were investigated for colonies on CROMagar STEC medium. Bacteria producing mahogany-color colonies were tested with ELISA. The SHIGA TOXIN CHEK (Alere, USA) was used as the micro ELISA kit. The kit contain 96 wells micro ELISA plates covered with monoclonal antibodies against Stx1 and Stx2. Results were read at 450/620 nm wavelength with a spectrophotometer device (μQuant Microplate Spectrophotometer, BioTek, USA). Samples with OD >0.080 were accepted as positive.

DNA Isolation

Multiplex PCR was applied to DNA samples isolated from both MacConcey broth and mahogany colored colonies growht on the CROMagar STEC.

From MacConkey broth, 500 μl was taken and centrifuged at 10000 rpm for 5 minutes. Precipitate at the base of the tube had 500 μl sterile water added. Then it was boiled for 10 minutes at 100 °C and the tube was cooled in ice for 10 minutes. Tubes were centrifuged for 3 minutes at 14000 rpm. After the centrifuge procedure, 100 μl of the fluid remaining at the top of the tube was removed and stored in a freezer at -30 °C for use as template DNA. Nearly 60 ng DNA was detected in 1 μl of the supernatant obtained by isolation (Nano-200 Micro Spectrophotometer, China). (Hala and Ehab2010; Sánchez et al. 2010). All procedures were made for each samples.

DNA Isolation from EMB medium: The mahogany-colored colonies growht on the CROMagar STEC medium were passaged on to eosin methylene blue (EMB) medium. Based on the colony size on EMB medium, 3-5 colonies were transferred to tubes containing 100 μl sterile distilled water. The tubes were boiled for 10 minutes at 100 °C and cooled. Then tubes were centrifuged for 2 minutes at 13000 rpm and the supernatant fluid above the precipitate at the base of the tube was stored at -30 °C to be used as a template DNA. (Dastmalchi and Ayremlou 2012)

PCR

For investigate *stx1, stx2, HlyA, eae, 16 srRNA* gene regions multiplex pcr assay were used for each DNA isolates from the MacConcey broth and mahogany-colored colonies growht on the CROMagar. For the amplification procedure, a 50 µl PCR mixture was prepared. The PCR reaction was performed in a BIO-RAD ICycler Thermal Cycler. *E. coli* ATCC 43895 strain was used for positive control and *E. coli* ATCC 25922 strain was used for negative control. For the PCR mixture, 10X Taq buffer 5 µl (100 mM Tris-HCl, 500 mM KCl:Thermo Scientific, USA), 1.5 mM $MgCl_2$ (3 µl, 25 mM $MgCl_2$: Thermo Scientific, USA), 0.2 mM dNTPs (5 µl, 2mM dNTPs: Thermo Scientific, USA), forward primer 2 µl (10 pikomol.μl^{-1}: Thermo Scientific, USA), reverse primer 2 µl (10 pikomol.μl^{-1}: Thermo Scientific, USA) (Table 1), Taq DNA polymerase: 1 µl (5U: Thermo Scientific, USA), template DNA 2 µl and sterile distilled water to reach 50 µl were used.

For initial denaturation at 94 °C for 4 minutes;

40 cycles,

- 94°C (1 min denaturation),
- 48°C (1 min adhesion),
- 72°C (90 s lengthening)

After the last cycle ended, final lengthening was performed with 5 minutes incubation at 72 °C.

Table 1. Sequences used as primers and predicted length of amplification products (Schmidt et al., 1995; Wang et al., 2002; Blanco et al., 2003)

Gene region	Oligonucleotide sequence	Length of amplification product
Stx1	R:5'-CGT GGT ATA GCT ACT GTC ACC-3' F:5'-CGC TGA ATG TCA TTC GCT CTG C-3'	302 bp
Stx2	R:5'-CTG CTG TGA CAG TGA CAA AAC GC-3' F: CTT CGG TAT CCT ATT CCC GG-3'	516 bp
EHEC-HlyA	R:5'-TCT CGC CTG ATA GTG TTT GGT A-3' F:5'-GGT GCA GCA GAA AAA GTT GTA G-3	1551 bp
eae	R:5'-GCG GTA TCT TTC GCG TAA TCG CC-3' F:5'-GAG AAT GAA ATA GAA GTC GT-3'	775 bp
16S rRNA	R:5'-ACC GCT GGC AAC AAA GGA TA -3' F:5'-CCC CCT GGA CGA AGA CTG AC-3'	401 bp

R: Reverse, **F:** Forward, **bp:** base pair, ***Stx1*:** Shiga toxin 1; ***Stx1*:** Shiga toxin 2; **EHEC-*HlyA*:** Enterohemorrhagic *Escherichia coli* hemolysin A; ***eae*:** "Effacing and attaching"; **16S rRNA:** 16S Ribosomal ribonucleic acid.

Table 2. National Center for Biotechnology Information Access Numbers for positive polymer chain reaction samples

Sample Number	Positive Gene Region	NCBI Access Number
18	*eae*	KT009017
101	*Stx1*	KT009018
47	*Stx1*	KT009019
51	*Stx1*	KT009020
180	*Stx2*	KT009021
18	*Stx2*	KT009022
47	*Stx2*	KT009023
131	*Stx2*	KT009024
39	*Stx2*	KT009025
25	*Stx2*	KT009026

NCBI: National Center for Biotechnology Information, ***Stx1*:** Shiga toxin 1; ***Stx2*:** Shiga toxin 2; ***eae:*** "Effacing and attaching"

Electrophoresis and Assessment of Bands Procedure

Amplified PCR products underwent the electrophoresis procedure (Thermo scientific EC300 XL, USA) with 1.5% agarose gel (Prona, Spain) containing ethidium bromide at 100 V for 90 minutes. The electrophoresis procedure used a 100-1500 bp (GeneON, Germany) marker.

Bands forming as a result of electrophoresis were investigated with a gel imaging system (Gel Doc 2000, Bio-Rad, USA). Sequencing the amplified PCR products were sent to MedSanTek (Istanbul, Turkey). Analysis of the sequence results were performed using the DNA Chromatogram Explorer Lite V4.0.0 programe and compared with the NCBI-Nucleotide genebank database. Access numbers obtained for every gene region (Table 2).

Serotyping

The STEC colonies growht in medium and isolated were sent to the National Enteric Pathogens Reference Central Laboratory in the Turkish Public Health Institution in Ankara. Samples with agglutination to O and/or H antigens had serotypes identified and results were reported.

Statistical Analysis

Statistical analysis was performed using SPSS 21 with serial number 10240642 and Medcalc V14.12 statistical programs. Statistical analysis used the McNemar test and calculated sensitivity, specificity, positive and negative cut-off values. Descriptive statistics are given as arithmetic mean ± standard deviation. For all statistics, the limit of significance was chosen as $p<0.05$.

RESULTS

Cattle samples

Of 180 cattle feces, 11 (6.1%) were identified to be positive for Stx presence with the ELISA method. Of the 11 samples positive for Stx with the ELISA method, 8 (4.4% of the total sample number) were positive for 16S rRNA along with at least 1 positive for *Stx1, Stx2,* and *eae* gene regions with PCR (Figure 1) 6 were positive for S*tx2*, 3 for S*tx1*, and 1 was positive for *eae* with the PCR method. The number of samples positive for the *Stx2* gene region was more than for the *Stx1* region and the difference was statistically significant (p=0.0001). However, there was no statistically significant difference related to the detection rates for this pathogen between the ELISA and PCR methods (p=0.250). Colonies of three samples (1.6%) (Figure 2) were positive for the targeted pathogenicity regions with ELISA. Additionally, *E. coli* was confirmed by definition with the Vitek 2 Compact (Biomerieux, France) system and STEC was identified. Only sample no. 180 was serotyped as O103:NM, while the other 2 samples could not be serotyped (Table 3). Positive samples are summarized one-by-one with test results for each method in the table (Table 4). Of the 169 animal samples negative on the ELISA test, none of the targeted *Stx1, Stx2, eae, and EhlyA* gene regions were identified with PCR and they were accepted as negative in terms of STEC.

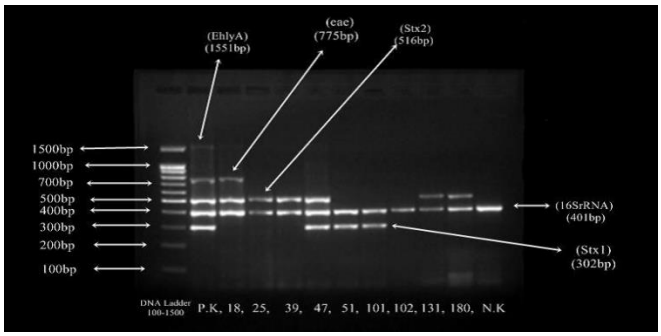

Figure 1: Appearance of agarose gel bands in sample positive according to ELISA test and polymerase chain reaction.
PK: positive control, **NK:** negative control.

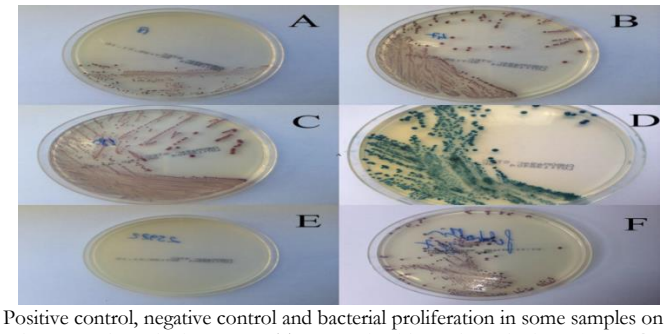

Figure 2: A-G. Positive control, negative control and bacterial proliferation in some samples on CROMagar STEC medium.
STEC positive mahogany-color colonies (A-C). STEC negative blue-color colonies (D). *E. coli* ATCC 25922 (no proliferation) (E). *E. coli* ATCC 43895 (STEC positive mahogany-color colonies) (F).

Table 3. Serotyping results for strains

No	NEPRL Protocol No	Laboratory Protocol No	Serotype
1	140583	Sample no. 18	ONT:NM
2	140584	Sample no. 47	ONT:NT
3	140585	Sample no. 180	O103:NM
4	140586	Positive control	O157:H7

NT: Non-typeable; **NM**: Non-motile **NEPRL**: National Enteric Pathogens Reference Laboratory

Table 4. Cattle feces samples with at least one positive result from the 3 methods

Sample No:	ELISA	PCR	Proliferation on CROMagarSTEC
13.	Positive	Negative	Negative
18.	Positive	Positive(16s rRNA, *Stx2, eae*)	Positive
25.	Positive	Positive (16s rRNA, *stx2*)	Negative
39.	Positive	Positive (16s rRNA, *Stx2*)	Negative
47.	Positive	Positive (16s rRNA, *stx1,Stx2*)	Positive
51.	Positive	Positive(16s rRNA, *Stx1*)	Negative
101.	Positive	Positive (16s rRNA, *Stx1*)	Negative
102.	Positive	Negative(only 16s rRNA positive)	Negative
129.	Positive	Negative	Negative
131.	Positive	Positive (16s rRNA, *Stx2*)	Negative
180.	Positive	Positive (16s rRNA, *Stx2*)	Positive

Stx1: Shiga toxin 1; ***Stx2***: Shiga toxin 2; ***eae***:"Effacing and attaching"; **16S rRNA:**16S ribosomal nucleic acid

Human samples

There were 143 men and 107 women among the 250 patients with fecal samples investigated. The general mean age was 23.2 years, with the mean age for women 25.2 years and mean age for men 21.2 years. Of the samples, 108 were obtained from the pediatric emergency clinic, 86 from the adult emergency clinic, and the remaining 56 samples from a variety of wards and clinics.

Direct microscopic examination of fecal samples found abundant leukocytes in 98 (39.2%), rare leukocytes in 42 (16.8%) and very rare leukocytes in 26 (10.4%). Of the total of 166 samples (66.4%) with leukocytes identified, 57 also had erythrocytes observed (22.8%). Macroscopic investigation found 161 of the samples (64.4%) had mucous and/or liquid appearance, while the remaining 89 samples (35.6%) had soft texture. Tests found negative for the presence of STEC by PCR, ELISA and culture methods. Of the samples, 25 were identified to have other enteric bacteria including 14 *Salmonella* spp., 10 *Campylobacter* spp. and 1 *Shigella* spp.

DISCUSSION

Cattle appear to act as a significant reservoir for STEC infections occurring in humans. STEC colonization of these animals may reach 60%, while studies generally found the average rate was 10-25%. Carriers of O157 serotype are very rare compared to these rates; for example, this serotype was found in 0-2.8% of milk and meat cattle in the United States of America (USA) (Harada et al. 2023).In studies about animals in Türkiye, a study encompassing investigation of 1000 water buffalo feces in Samsun isolated 38 *E. coli* O157:H7 strains (3.8%) (Nuhay and Gülhan 2017). A study by Ayaz et al. in 2014 in the Kırıkkale region obtained carcass swabs, rectoanal mucosal swabs and bile samples from 240 cattle and isolated *E. coli* O157:H7 in 6.3% of swab samples (Erol2016). Another study performed in the Afyonkarahisar region in February-August 2014 on 237 cattle feces samples found this rate was 4.6% by PCR method (Aslan et al. 2016). A study in Bursa in 2014 found STEC in 6.3% of cattle (Ahmed 2017). Contrary to the low positivity rates in other studies, it was reported that *E. coli* O157 was isolated in 13.6% (77 samples) of rectal swab samples taken from 565 cattle carcasses mainly from Hatay but also Adana, Kahramanmaraş, and Mersin (Aslantaş et al. 2006).

There is little research about identifying the STEC colonization rates in cattle in our country. When the available studies are examined, some had no positivity

identified while some detected high rates like 13.6% positivity. In our study, a total of 8 animals were found to be positive STEC carriers with both ELISA and PCR methods, though none of the 3 isolated STEC strains were found to be O157:H7 according to serotyping results. The difference in findings obtained as a result of studies is considered to be due to geographical differences, isolation and definition methods not being the same and seasonal differences.
The number of studies performed to determine the prevalence of STEC strains apart from O157 is at lower levels compared to O157, which is linked to the later understanding of the importance of these strains. The prevalence of non-O157 strains is generally ignored; however, non-O157 serotypes were isolated in 25% of people developing HUS. (WHO 1998). According to the European Food Safety Authority (EFSA) data for 2012, 3316 cattle were investigated for the presence of STEC and 195 were identified to be positive (5.9%). Of the 129 cattle samples that could be serotyped, 13 different O serotypes were found (EFSA 2022). In Türkiye, the studies to determine the prevalence of STEC in cattle were performed to identify the O157:H7 strains, as in many other countries. For this reason, the non-O157 STEC prevalence rates are not known. In this study, of the 3 strains isolated from 180 samples, only 1 (0.5%) was serotyped as O103:NM (non-motile). A study in 2013 from Greece investigated 140 cattle with both ELISA and PCR methods. They found 4 samples positive according to ELISA (2.9%) and 2 samples positive with PCR (1.4%). The 2 samples positive with PCR were serotyped as O157:H7. In the study, they emphasized that the PCR method was a more reliable method to identify STEC (Pinaka et al. 2013).
In our study, 8 out of 11 samples positive according to ELISA were identified to have *Stx1* and/or *Stx2* gene regions with PCR, while the other 3 samples were negative. It is considered that the ELISA method provided false positive results for the 3 negative samples. Different studies have stated that ELISA tests may provide false positive results (Ball et al. 1996; Pulz et al.2003). The sensitivity, specificity, positive and negative predictive values for the ELISA test compared to PCR were 100%, 98.26%, 72.73% and 100%, respectively. In our study, among 180 animal feces, the 8 samples consistent with STEC that were positive with PCR and also had sequencing results compared to the database is equivalent to 4.4%. The studies performed in Türkiye to determine STEC prevalence mainly used SMAC medium and defined the strains according to whether O157 agglutinated with antiserum by choosing sorbitol-negative colonies. For this reason, most research only provides information about the frequency of O157. There is not much data about both the O157 and non-O157 STEC colonization rates. In this study, instead of SMAC agar or CT-SMAC agar, STEC strains apart from O157 were produced using the CROMagar STEC medium, with differentiation ability. However, only 3 of the 8 samples positive for STEC with PCR (38%) could be isolated from this medium. This difference is thought to be due to the low STEC bacteria counts in the inoculated samples and lack of growth of this bacteria in the medium, even though PCR may identify positive samples by providing sensitive results in the presence of very little bacteria.
The incidence of STEC in humans is different from country to country. According to CDC FoodNet data, the STEC incidence in the USA was reported as 1.5, 1.69 and 1.81 for the years 2015, 2016 and 2017 representing 4824, 5443 and 6034 cases, respectively (CDC 2021). According to EFSA data, the STEC incidence was 1.9, 1.5 and 2.1 for 2019, 2020 and 2021, respectively. For the same years, 7801, 4489 and 6084 cases were reported (EFSA2022). Studies of humans in our country, similar to animal studies, dominantly used SMAC medium and chose sorbitol-negative colonies with detection according to agglutination with O157 antiserum. For this reason, most research only provides information about the O157 strains. The results of different studies in Türkiye found the STEC O157:H7 incidence was between 0 and 4% (Ünlü 2015).
It is known that non-O157 serotypes were identified in some studies in recent years. In 2011 an epidemic due to O104:H4 serotype occurred in Europe, led by Germany, and caused 3816 cases with 845 of these cases (22%) developing HUS and the death of 54 patients reported (Frank et al. 2011). The effect of this epidemic in Türkiye was an increase in HUS in pediatric patients in the same year. Of 70 patients with HUS diagnosis treated in a total of 40 pediatric centers, only 4 were serotyped and 2 were O104:H4 (Ekinci at al. 2013). A study of fecal samples obtained from children with suspected HUS from 2012 to 2019 identified STEC in 46 patients. Of these 15 were O145 serotype (32.6%) and 8 were O157 serotype (17.3%) (Okumuş 2021). A study investigating 395 samples sent to the Public Health Reference Laboratory for suspected HUS from 2011 to 2014 identified STEC in 28 samples. Among these samples, the dominant serotypes were O104 for 7 samples and O26 for 6 samples (Gulesen et al.2016). In our study, none of the 250 fecal samples from humans had O157:H7 or non-O157 STEC strains identified. The reason for this may be the low prevalence of bacteria in the region, probable antibiotic use and patients attending hospital late after diarrhea begins. In fact, it is reported that the rate of identification of this bacteria reduces as time passes after diarrhea begins (Rosensweig andGourley 1991).

CONCLUSION

For the detection of STEC, due to the difficulty in isolating this bacteria with culture methods and the high cost of ELISA tests and the antiserums used in the agglutination method, the use of molecular methods in future research will be both rapid and more

beneficial. With this aim, the use of the multiplex PCR method can be said to be appropriate in terms of both sensitivity and specificity. Additionally, serotyping is necessary for bacteria in positive samples for gene regions causing virulence, especially in epidemiological studies. As this study is the first and only study in the region, there is a need to perform more studies on both human and animal samples to determine prevalence.

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RESEARCH ARTICLE

Characterization of Anogenital Distance and Its Relationship with Fertility in German Fawn × Hair Crossbred and Saanen Goats

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ABSTRACT

In the presented study, it was aimed to reveal the characterization of anogenital distance (AGD) and its relationship with fertility in German Fawn × Hair crossbred and Saanen goats whose estrus was synchronized during the breeding season. 53 heads of German Fawn × Hair crossbred (75% German Pied and 25% Hair goat) and 55 heads of Saanen breed goats were used in the study. Before applying the sponge, the anogenital distances of all goats were measured and recorded with a digital caliper. For each breed, they were divided into two AGD categories: short AGD group and long AGD group. A sponge containing 60 mg medroxyprogesterone acetate was applied intravaginally to these goats on day 0, and the sponges were kept in the vagina for 13 days. On the thirteenth day of application, sponges were removed in all goats, and eCG was administered at a dose of 500 IU at the time of sponge removal. Goats on estrus were hand mated with one of the proven bucks (goat:buck ratio of 7:1). The parameters of estrus rate, pregnancy rate, kidding rate, multiple birth rate, litter size and male litter rate were calculated in all groups. After the statistical analysis, no significant difference was detected in the parameters monitored between the groups (p>0,05). It was concluded that anogenital distance did not make a difference on fertility in German Fawn × Hair crossbred and Saanen goats whose estrus was synchronized with progestagen applications during the breeding season. It is thought that more detailed studies are needed to clearly demonstrate the characterization of anogenital distance and its relationship with fertility in goats.

Keywords: Anogenital distance, breeding season, fertility, goat

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Alman Alaca × Kıl melezi ve Saanen Irkı Keçilerde Anogenital Mesafenin Karekterizasyonu ve Fertilite ile İlişkisi

ÖZ

Sunulan çalışmada, üreme mevsimi içerisinde östrüsları senkronize edilen Alman Alaca × Kıl melezi ve Saanen ırkı keçilerde anogenital mesafenin (AGM) karekterizasyonu ve fertilite ile ilişkisinin ortaya konulması amaçlanmıştır. Çalışmada 53 baş Alman Alaca x Kıl melezi (%75 Alman Alaca ve %25 Kıl keçisi) ve 55 baş Saanen ırkı keçi kullanıldı. Sünger uygulanmadan önce tüm keçilerin anogenital mesafeleri dijital kumpasla ölçülerek kaydedildi. Her ırk için kısa AGM grubu ve uzun AGM grubu olmak üzere iki AGM kategorisine ayrıldı. Bu keçilere 0. gün intravajinal 60 mg medroksiprogesteron asetat içeren sünger uygulandı, süngerler 13 gün süreyle vajinada tutuldu. Tüm keçilerde uygulamanın on üçüncü günü süngerler çıkarıldı ve sünger çıkarılması anında 500 IU dozda eCG uygulandı. Östrüste olduğu tespit edilen keçiler elde aşım yöntemiyle (teke/keçi oranı: 1/7) fertil tekelerden bir tanesi ile çiftleştirildi. Teke katımından sonraki 40. günde çiftleşen hayvanlara ultrasonografik gebelik muayenesi yapıldı. Tüm gruplarda östrüs oranı, gebelik oranı, doğum oranı, çoklu doğum oranı, yavru verimi ve erkek yavru oranı parametreleri hesaplandı. Yapılan istatistiksel analiz sonrası gruplar arasında takip edilen parametreler arasında önemli bir fark saptanmadı (p>0,05). Sonuç olarak üreme mevsimi içerisinde progestagen uygulamaları ile östrüsları senkronize edilen Alman Alaca x Kıl melezi ve Saanen ırkı keçilerde anogenital mesafenin fertilite üzerine farklılık yaratmadığı görüldü. Keçilerde anogenital mesafenin karekterizasyonu ve fertilite ile ilişkisinin açıkça ortaya konulabilmesi için daha ayrıntılı çalışmalara ihtiyaç olduğu düşünülmektedir.

Anahtar Kelimeler: Anogenital mesafe, fertilite, keçi, üreme sezonu

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GİRİŞ

Reprodüktif karakteristiğe ilişkin belirteçlerin kullanılması, fertiliteye yönelik genetik iyileştirmelerin hızını ve etkinliğini potansiyel olarak artırabilmektedir (Grala ve ark. 2021). Teknoloji ve bilim alanlarındaki gelişmeler sayesinde, çok sayıda belirteç ile hayvanların yalnızca genotip bilgileri kullanılarak, genomik seleksiyon yöntemiyle çiftlik hayvanlarında yapılan genom analizleri ve tek nükleotid farklılıkları (Single Nucleotide Polymorphism/SNP) çip teknolojisi ile son 10 yılda yaygınlaşarak uygulanabilir hale gelmiştir (Özkan ve Yakan 2017). Ancak genomik seleksiyonda kullanılan belirteçlerin tespiti, maliyeti ve farklı türlerde ve aynı türün farklı ırklarında çeşitlilik sağlaması dezavantaj yaratmaktadır (Özkan ve Yakan 2017). Bu durum çiftlik hayvanlarında fertiliteye ilişkin ucuz ve güvenilir belirteçlerin arayışına sebep olmuştur. Endüstriyel hayvancılıkta anogenital mesafe (AGM)'nin fertilite ile ilişkilendirilmesi Gobikrushanth ark. (2017) tarafından süt ineklerinde bildirilmiştir. Anogenital mesafe, kadınlarda anüsün merkezinden klitorisin tabanına (Salazar-Martinez ve ark. 2004) veya klitorise (Sathyanarayana ve ark. 2010) kadar, süt ineklerinde ise anüsün merkezinden klitorisin tabanına kadar olan ölçüm olarak tanımlanmaktadır (Gobikrushanth ve ark. 2017). Dişi fetüslerin uterusta prenatal gelişimi sırasında aşırı androjen maruziyeti üreme sisteminin androjenizasyonuyla sonuçlanmaktadır (Bowman ve ark. 2003). Bu androjenizasyon fareler (Zehr ve ark. 2001), tavşanlar (Bánszegi ve ark. 2012), domuzlar (Drickamer ve ark. 1997) ve kadınlar (Mendiola ve ark. 2012; Mira-Escolano ve ark. 2014; Wu ve ark. 2017) dahil olmak üzere çeşitli türlerde AGM'nin artmasına ve olumsuz fertilite sonuçlarına yol açtığı bildirilmiştir. Ancak önceki yapılan çalışmaların aksine Murciano-Granadina ırkı keçilerde yapılan çalışmada ise AGM ile fertilitenin doğru orantılı olduğu bildirilmiştir (Shourabi ve ark. 2022).

Mevcut literatürde keçilerde anogenital mesafe ile gebelik oranları arasındaki ilişkiyi inceleyen sınırlı sayıda çalışma bulunmaktadır. Bu nedenle sunulan çalışma ile üreme mevsimi içerisinde progestagen uygulamaları ile östrüsları senkronize edilen Alman Alaca x Kıl melezi ve Saanen ırkı keçilerde anogenital mesafenin karekterizasyonu ve fertilite üzerine etkilerinin ortaya konulması amaçlanmıştır. Bu sayede keçilerde AGM'nin basit morfolojik ölçümü ile üreme performansı arasındaki ilişki doğrulanabilirse AGM, keçiler için gelecekteki genetik seleksiyon programlarında kullanılacak yeni bir üreme fenotipi haline gelebileceği öngörülmektedir.

MATERYAL ve METOT

Hayvan Materyali

Çalışma Ağustos 2023 tarihinde Adana ili Sarıçam ilçesinde Çukurova Üniversitesi Ziraat Fakültesi Araştırma ve Uygulama Çiftliği Keçicilik işletmesinde yürütüldü. Çalışmada bir önceki sezonda doğum yapmış, genital organlarında klinik sorun belirlenmeyen, 2-5 yaş aralığında, sağlıklı, 45-50 kg canlı ağırlıktaki 53 baş Alman Alaca x Kıl melezi (%75 Alman Alaca ve %25 Kıl keçisi) ve 55 baş Saanen ırkı keçi kullanıldı. Tüm keçiler %60 kaba yem ve %40 kesif yemden oluşan standart rasyonla beslendiler. Kaba yem kaynağı olarak sırasıyla %75 ve %25 olmak üzere mısır silajı ve yonca samanı kullanıldı. Rasyonlar, OptiTMR Pro 4.0.33 kullanılarak keçilerin enerji ve protein gereksinimlerini karşılayacak şekilde formüle edildi. Suya ulaşımları 24 saat boyunca ad-libitum sağlandı. Aşım öncesi hayvanlara flashing uygulaması yapılmadı. Çalışma Çukurova Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'nun 20.07.2023 tarih ve 5/14 sayılı onayı ile gerçekleştirildi.

Anogenital Mesafenin Belirlenmesi

Keçilere sünger uygulanmadan önce anüsün merkezinden klitorisin tabanına kadar olan mesafe aynı kişi tarafından dijital kumpas (Piranha, Çin) ile ölçüldü. Shourabi ve ark. (2022) ve Feknous ve ark. (2021)'nın çalışmalarında belirtildiği gibi, her ırk (Alman Alaca x Kıl melezi "A" ve Saanen "S") için kısa AGM grubu (çalışmadaki keçiler AGM ≤ AGM medyanı) ve uzun AGM grubu (çalışmadaki keçiler AGM > AGM medyanı) olmak üzere iki AGM kategorisine ayrıldı.

Östrüs Uyarımı

Tüm keçilere 13 gün süreli 60 mg medroksiprogesteron asetat içeren intravaginal sünger (Esponjavet®, Hipra, İspanya) uygulandı (0. gün). Süngerin çıkarıldığı gün (13. gün) 500 IU dozda eCG (At koryonik gonodotropini, Oviser®, Hipra, İspanya) kas içi olarak yapıldı. Östrüs tespitine süngerlerin çıkarılmasından 12 saat sonra başlandı ve östrüs tespiti sabah ve akşam (06.00-07.00:18.00-19.00) saatlerinde arama tekesi ile yapıldı. Östrüste olduğu tespit edilen keçiler elde aşım yöntemiyle (teke/keçi oranı: 1/7) fertil tekelerden bir tanesi ile çiftleştirildi. Elde aşım bittikten sonra tekeler sürüden çıkartıldı.

Ultrasonografik Muayene

Ultrasonografik muayenelerde real-time B-mode ultrason cihazı (Hitachi EUB-405, 3,5 MHz konveks prob, Japonya) kullanıldı. Aşım sonrası 40. günde uygulanan transabdominal gebelik muayenesinde gebelik kesesi, embriyo/fetüs'ün varlığı, yavru sıvıları ve plasentomların görülmesi halinde keçiler gebe olarak değerlendirildi.

Üreme Parametrelerinin Değerlendirilmesi:

Oğlak sayıları keçilerin doğumunu takiben not edildi ve üreme parametreleri olarak östrüs oranı, gebelik oranı, doğum oranı, çoklu doğum oranı, yavru verimi

ve erkek yavru oranı aşağıda belirtilen formüllerden faydalanılarak hesaplandı:

$$\text{Östrüs Oranı} = \frac{\text{östrüs gösteren keçi sayısı}}{\text{intravajinal sünger takılan keçi sayısıı}} \; x\,100$$

$$\text{Gebelik Oranı} = \frac{\text{gebe keçi sayısı}}{\text{intravajinal sünger sonrası östrüs gösteren ve aşım yapılan keçi sayısı}} \; x\,100$$

$$\text{Doğum Oranı} = \frac{\text{doğum yapan keçi sayısı}}{\text{intravajinal sünger sonrası gebe kalan keçi sayısı}} \; x\,100$$

$$\text{Çoklu Doğum Oranı} = \frac{\text{ikiz} + \text{üçüz} + \text{dördüz doğum yapan keçi sayısı}}{\text{doğum yapan keçi sayısı}} \; x\,100$$

$$\text{Yavru Verimi} = \frac{\text{toplam doğan oğlak sayısı}}{\text{doğum yapan keçi sayısı}}$$

$$\text{Erkek Yavru Oranı} = \frac{\text{erkek oğlak sayısı}}{\text{toplam oğlak sayısı}} \; x\,100$$

İstatistiksel Analiz

Elde edilen verilerin istatistiksel karşılaştırılmasında SAS Versiyon 8.0 paket programından yararlanıldı. Her ırkın alt grupları arasında üreme parametrelerinin (oransal verilerin) karşılaştırılmasında Ki-kare testi ve Fisher'ın Tam testi, PROC GENMOD prosedürü kullanıldı. Hesaplanan p değerlerinin 0,05'den küçük olması durumunda önemli olarak kabul edildi.

BULGULAR

Keçilerde anogenital mesafe için tanımlayıcı istatistikler Tablo 1.'de sunuldu. Tüm gruplarda üreme parametrelerine ilişkin elde edilen sonuçlar Tablo 2.'de sunuldu. Östrüs oranı, gebelik oranı, doğum oranı, çoklu doğum oranı, yavru verimi ve erkek yavru oranı parametreleri yönünden yapılan istatistiksel analizde gruplar arasında önemli bir fark saptanmadı (p>0,05)

Tablo 1. Keçilerde anogenital mesafe için tanımlayıcı istatistikler

Table 1. Descriptive statistics for anogenital distance in goats

Gruplar		n	Medyan (mm)	Ortalama (mm)	Standart Hataların Ortalaması (mm)	En küçük (mm)	En büyük (mm)
Alman Alaca x Kıl Melezi		53	43,1	44,01	0,73	33,13	61,82
	A-Kısa AGM	27	40,61	39,96	0,52	33,13	43,1
	A-Uzun AGM	26	47,5	48,22	0,757	43,6	61,82
Saanen		55	47,49	47,42	0,665	37,48	58,69
	S-Kısa AGM	28	44,1	43,56	0,572	37,48	47,49
	S-Uzun AGM	27	50,98	51,41	0,561	47,5	58,69

Tablo 2. Gruplarda üreme parametreleri

Table 2. Reproductive parameters in groups

	Alman Alaca x Kıl Melezi		**Saanen**	
Gruplar	A-Kısa AGM (n=27)	A-Uzun AGM (n=26)	S-Kısa AGM (n=28)	S-Uzun AGM (n=27)
Östrüs Oranı	%100 (27/27)	%100 (26/26)	%100 (28/28)	%100 (27/27)
Gebelik Oranı	%81,5 (22/27)	%73,1 (19/26)	%85,7 (24/28)	%77,8 (21/27)
Doğum Oranı	%100% (22/22)	100% (19/19)	%100 (24/24)	%100 (21/21)
Çoklu Doğum Oranı	%95,5 (21/22)	%84,2 (16/19)	%87,5 (21/24)	%81,0 (17/21)
Yavru Sayısı	50	42	64	50
Tek	1 (1)	3 (3)	3 (3)	4 (4)
İkiz	14 (28)	9 (18)	7 (14)	7 (14)
Üçüz	7 (21)	7 (21)	9 (27)	8 (24)
Dördüz	-	-	5 (20)	2 (8)
Yavru Verimi	2,27 (50/22)	2,21 (42/19)	2,67 (64/24)	2,38 (50/21)
Erkek Yavru Oranı	%56 (28/50)	%54,8 (23/42)	%51,6 (33/64)	%54,0 (27/50)

*Yapılan istatiksel analizde gruplar arasında önemli bir fark saptanmadı. (p>0,05)

TARTIŞMA

Anogenital mesafenin prenatal androjenizasyonun bir belirteci olduğu tespit edilmiştir (Macleod ve ark. 2010; Dean ve ark. 2012). Prenatal dönemde yüksek seviyedeki testesteron konsantrasyonu dış cinsel organlarda erkekleşmeye yol açabileceği ve uzayan AGM ile sonuçlanabileceği gösterilmiştir (Manikkam ve ark. 2004; Hotchkiss ve ark. 2007; Lamm ve ark. 2012).

Sunulan çalışmada östrüs oranı tüm gruplarda %100 olarak tespit edilmiş, istatistiksel olarak gruplar arasında fark olmadığı görülmüştür. Geçiş sezonunda (temmuz) Alman alaca x kıl melezi keçilerde uzun süreli (17 gün) progestagen + eCG (400 IU) + dinoprost tromethamine (12,5 mg) uygulanan bir çalışmada keçilerin östrüs oranları %100 olarak tespit edilmiştir (Kutlu ve ark. 2022). Sunulan çalışmada elde edilen bu sonuç önceki çalışmada (Kutlu ve ark. 2022) aktarılan değerle uyum sağlayarak östrüs oranlarının benzer olduğu görülmektedir.

Sunulan çalışmada gebelik oranlarının ırklar arasında ve gruplar arasında istatistiksel açıdan farklı olmadığı (p>0,05), AGM'nin gebelik oranı üzerine herhangi bir olumlu ya da olumsuz etkisi olmadığı tespit edildi. Sığırlarda yapılan önceki çalışmalarda, kısa AGM gruplarında uzun AGM gruplarına göre gebelik oranını; ilk laktasyondaki Kanada Holştaynlarında +%22,7 (Gobikrushanth ve ark. 2017), ilk laktasyondaki Holştaynlarda +%10 (Grala ve ark. 2021), nullipar Holştaynlarda +8,7% (Carrelli ve ark. 2021), ve Kuzey Amerika Holştaynlarında +%6 (Carrelli ve ark. 2022) oranlarında istatistiksel olarak artırdığı, Kanada Holştaynlarında +%16,1 (Gobikrushanth ve ark. 2017) ve İran Holştaynlarında +18,6% (Akbarinejad ve ark. 2019) artırma eğiliminde olduğu, ikinci laktasyondaki Holştaynlarda (Grala ve ark. 2021), İrlanda Holştaynlarında (Gobikrushanth ve ark. 2019), Belçika ve Hollanda Holştaynlarında (Beci ve ark. 2023) istatistiksel olarak değişiklik

yaratmadığı bildirilmiştir. Bu çalışmalardan farklı olarak Makiabadi ark. (2022) Holştayn ineklerde AGM'yi 3 gruba ayırmışlar postpartum ilk tohumlamada konsepsiyon oranı orta AGM grubunda kısa ve uzun AGM gruplarına göre daha yüksek (sırasıyla %46, %24 ve %28) olarak tespit etmişlerdir. Sığırlarda yapılan çalışmaların aksine Shourabi ark. (2022), Murciano-Granadina ırkı keçilerde gebelik oranının uzun AGM grubunda kısa AGM gruplarına göre %27,5 daha yüksek olduğunu bildirmiştir (sırasıyla %85,3 ve %57,8). Sunulan çalışmada elde edilen bu sonuçlar bazı (Gobikrushanth ve ark. 2019; 2021; Beci ve ark. 2023) çalışmalardan aktarılanlarla uyum sağlarken bazılarıyla da (Gobikrushanth ve ark. 2017; Akbarinejad ve ark. 2019; Carrelli ve ark. 2021; Grala ve ark. 2021; Carrelli ve ark. 2022; 2022) sağlamamaktadır. Çalışmalarda elde edilen farklı sonuçlar türlerin ve ırkların farklılığından kaynaklanıyor olabilir.

Yavru verimi ekonomik olarak karlılığı belirleyen önemli üreme parametrelerinden biridir. Sunulan çalışmada yavru veriminin ırklar arasında ve gruplar arasında istatistiksel açıdan farklı olmadığı ($p>0,05$) tespit edilmiştir. Önceki yapılan çalışmalarda Feknous ve ark. (2021) tavşanlarda yavru verimini (8,96'e karşı 7,83) ve erkek yavru oranını (%61,6'e karşı %41) kısa AGM grubunda uzun AGM grubuna göre daha yüksek olduğunu bildirmişlerdir. Aksine Shourabi ve ark. (2022) keçilerde uzun AGM grubunda yavru verimini ve erkek yavru oranının (%53,8'e karşı %42,2) kısa AGM grubundan daha yüksek olduğunu bildirmiştir ($p<0,05$). Sunulan çalışmada elde edilen bu sonuçlar önceki çalışmalarda aktarılan sonuçlarla uyum sağlamamaktadır. AGM'nin yavru verimi ve erkek yavru oranı üzerine şu anda sınırlı bilgi mevcut olduğundan bu ilişkinin aydınlatılması için daha fazla sayıda ve farklı keçi ırklarında daha fazla çalışmaya ihtiyaç duyulduğu görülmektedir.

SONUÇ

Sonuç olarak üreme mevsimi içerisinde progestagen uygulamaları ile östrüsları senkronize edilen Alman Alaca x Kıl melezi ve Saanen ırkı keçilerde kısa AGM gruplarında rakamsal olarak gebelik oranı, çoklu doğum oranı, yavru verimini yüksek olduğu tespit edilsede istatistiksel olarak farklılık yaratmadığı görüldü. Keçilerde anogenital mesafenin karakterizasyonu ve fertilite ile ilişkisinin açıkça ortaya konulabilmesi için daha ayrıntılı çalışmalara ihtiyaç olduğu düşünülmektedir.

Çıkar çatışması: Bu çalışmada çıkar çatışması bulunmamaktadır.

Yazarların Katkı Oranı: Yazarlar makaleye eşit oranda katkı sağlamış olduklarını beyan etmişlerdir.

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RESEARCH ARTICLE

Environmental Factors Affecting Economically Important Traits of Anatolian Buffalo in Yozgat

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ABSTRACT

The aim of the study was to investigate the effects of some environmental factors on the growth, reproduction and production traits of Anatolian Buffaloes. The reproduction and production data of 1139 Anatolian buffaloes and the growth records of the calves between 2015 and 2019 in Yozgat province were used. The least-square means of the birth (BW), weaning (WW), sixth month (SMW) and yearling weights (YW) and daily gain between those traits were determined as 30.43, 97.79, 112.98, 169.40, 0.441, 0.459, 0.382 and 0.306 kgs respectively. Calving interval (CI) and service period (SP) were 470.08 and 150.08 days. Lactation milk yield (LMY), milk yield per day of lactation period (MY/LP), milk yield per day of CI (MY/CI), peak yield (PY), day at piek yield (DPY), and persistence (P) were found to be 860.40, 4.447, 1.916, 5.589 kgs, 83.34 days, and 77.35%. The effects of village and sex on BW, WW, SMW and YW were statistically significant ($P<0.05$) but the season affected all of these traits except for BW. Analysis of variance revealed all the environmental factors were significant on CI and SP. MY/LP and MY/CI weren't affected only by the season of calving and calving year respectively. The rest of the production traits were affected by all environmental factors. It was concluded that significant environmental factors such as the village, year, season, and age of the dam must be considered in farm management activities to improve the performances of Anatolian buffaloes.

Keywords: Anatolian buffalo, Economic traits, Environmental factors, Yozgat

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Yozgat İlinde Yetiştirilen Anadolu Mandalarında Ekonomik Özellikleri Etkileyen Çevresel Faktörler

ÖZ

Bu çalışma, Anadolu Mandalarının büyüme, üreme ve üretim özellikleri üzerine çevresel faktörlerin araştırılması amacıyla yapılmıştır. Bu amaçla Yozgat'ta 2015-2019 yılları arasında 1139 baş Anadolu mandasının üreme ve üretim verileri ile bunlardan doğan malakların büyüme verileri kullanılmıştır. Bu özellikler arasında doğum, sütten kesim, altıncı ay ve bir yaş ağırlığı ile bu özellikler arasındaki ortalama günlük canlı ağırlık kazancına ait en küçük kare ortalamaları sırasıyla 30,43; 97,79; 112,98; 169,40; 0,441; 0,459; 0,382 ve 0,306 kg'dır. Malaklama aralığı için en küçük kareler ortalaması 470,08 ve servis periyodu 150,08 gündür. Laktasyon süt verimi, laktasyonda ortalama günlük süt verimi, malaklama aralığında ortalama günlük süt verimi, pik verimi, pike ulaşım süresi ve süt veriminde inişe karşı direnme gücü en küçük kare ortalamaları 860,40; 4,447; 1,916 kg, 83,34 gün, 5,589±0,116 kg ve %77,35 olarak bulunmuştur. Doğum, sütten kesim, altıncı ay ve bir yaş ağırlığı üzerinde köy ve cinsiyetin etkisi istatistiksel olarak anlamlı bulunmuş ancak doğum ağırlığı hariç tüm bu özellikler mevsimden etkilenmiştir. Varyans analizi ayrıca tüm çevresel faktörlerin malaklama aralığı ve servis periyoduna anlamlı etkisi olduğunu ortaya çıkardı. Laktasyonda ortalama günlük süt verimini sadece buzağılama mevsimini etkilemezken, malaklama aralığında ortalama günlük süt verimini malaklama yılından etkilenmemiştir. Diğer süt verim özellikleri ise tüm çevresel faktörlerden etkilemiştir. Anadolu mandalarında verimlerin arttırılması için bakım ve idarede köy, yıl, mevsim ve ana yaşı gibi önemli çevresel faktörlerin dikkate alınması gerektiği sonucuna varılmıştır.

Anahtar Kelimeler: Anadolu mandası, Çevresel faktörler, Ekonomik özellikler, Yozgat

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INTRODUCTION

When the number of buffaloes is taken into account, buffalo breeding has kept its importance until recently in Türkiye. While the number of buffaloes was stated with millions in the 1970s, dropped to its lowest level in 2007. As a threatened animal, this breed was registered as an animal genetic resource and taken under protection in 2004. Protection was insufficient when the situation reassessment of the breed was due to its increasing commercial value. The Ministry of Agriculture and Forestry initiated a Community Based Anatolian Buffalo breeding project in 2011 (Kaplan et al. 2015). The importance of the Anatolian buffalo increases due to adaptation to environmental conditions, resistance to diseases, and the value of the products obtained from its meat and milk as well as the use of low quality roughage (Tekerli 2016; Soysal et al. 2018). In Türkiye, there are approximately 162000 buffaloes, which is one of the most important domestic animals in terms of genetic resources of the country (TUİK). Yields can be increased by improving the care, feeding and environmental factors of buffaloes. However, buffalo breeders to make plans for the future they need to know information about the yield levels of the animals in the aspect of effecting environmental factors. In addition, it is necessary to know both the traits on which selection is directed and significant factors to eliminate for calculating best breeding values. The aim of the study was to reveal the growth, reproductive, and productive traits of buffaloes and the environmental factors affecting them.

MATERIALS and METHODS

The data were obtained from the sub-project carried out in Yozgat province under the Community Based Anatolian Buffalo Breeding Project. The reproductive and productive data belonging to 1139 Anatolian buffaloes and growth records of their calves were used. BW, WW, SMW, YW, and daily gain among these traits are the growth parameters in the study. The reproductive parameters are CI and SP.

The production parameters are LMY, MY/LP, MY/CI, DPY, PY and P. The data provided by Manda Yılıdızı (Tekerli 2019), were used and controlled. Lactation lengths of less than 100 days and more than 365 days were not taken into account. The formula reported by Aziz et al. has utilized to calculate SP. In calculating the persistency, the modified coefficient of variation was used (Tekerli et al. 2001). Yozgat province is in the Central Anatolia Region and located between 34°05'-36°10' east meridians and 38°40'-40°18' north parallels. It is at an average altitude of 1300 meters above sea level and 15th among 81 provinces in terms of soil size. The basic economy of Yozgat is based on agriculture and animal husbandry. Villages and farms raising Anatolian buffaloes are distributed in 5 different counties, namely Akdağmadeni, Çekerek, Kadışehri, Merkez and Sorgun. Most of the buffalo breeders are engaged in both animal and plant production. The buffaloes are grown based on pasture by giving concentrated feed at different levels. Buffalo cows are inseminated naturally by bulls. Approximately 42% of the farms produce all or part of the feed they need. The average daily feed consumption of buffaloes consists of 9 kg forage and 4 kg concentrate. Buffalo cows spend half of the year as tied up in farms and the remaining half of the year is passed by grazing on the pasture all day. Milking is generally carried out by hand in the period between April and October (Kaplan et al. 2018). The least squares analysis was performed using the general linear model option of the Minitab (Minitab 2017). The significance levels of the differences between the groups were determined according to the TUKEY and FISHER. Statistical models used for the growth, reproduction and production traits are given below in their respective order.

$$Y_{ijklmno} = \mu + V_i + BY_j + BS_k + S_l + DA_m + AW_n + e_{ijklmno}$$ **Model [1]**

$$Y_{ijklm} = \mu + V_i + CY_j + CS_k + DA_l + e_{ijklm}$$ **Model [2]**

$$Y_{ijklmn} = \mu + V_i + CY_j + CS_k + DA_l + LP_m + e_{ijklmn}$$ **Model [3]**

Where; Y_{ijklmn}=observation, μ=overall mean, V=village, BY=birth year, BS=birth season, S=sex, DA=dam age, AW=age of weaning, CY=calving year, CS=calving season, LP=lactation period, $e_{ijklmno}$=error N (0, σ^2).

RESULTS

The least square means of the growth traits of calves different ages, reproduction and production traits of the cows and factors are presented in Table 1, 2 and 3. The village was significant (P<0.001) for all traits, showing that the changes in the care and feeding practices in the growing site could affect the growth and yield at a high level. The effect of the year was observed in birth and sixth month weights only (P<0.001). On the other hand, the season was found to be significant in the sixth month and one-year weights. It was determined that calves born in winter were heavier than those born in the other season. The sex had a significant effect on all traits and male calves were better than females in terms of weight. The least squares mean showed that only birth weight and DGSTM were affected by the age of dam from growth traits and calves born from cows older than seven years of age had higher birth weights than the others. Analysis of variance showed that the effect of weaning age on weight was significant (P<0.001).

This situation revealed that the breeders should care for calves to be suckled by their mothers. The significant (P<0.05; P<0.01; P<0.001) effects of village, year, and season on DGBSM and DGBTM indicated that these factors should be taken into consideration when determining the values of buffaloes in terms of these traits.

The reproductive traits were significantly (P<0.01; P<0.001) affected by environmental factors in ANOVA. The CI of cows delivered in the summer and autumn seasons was found to be longer. LMY was significantly (P<0.05; P<0.001) affected by all environmental factors. The fluctuations have shown significant differences in the care and feeding conditions according to the economic situations of the breeders, precipitation regime, drought, and so on. The highest milk yield was observed in cows that gave birth in the autumn and winter months. The least squares mean revealed that the buffaloes in Yozgat reached adult age yield in 7 or 10 years. The highest daily milk yield per day of calving interval was in the winter calvers showing that the most economical production is also realized in this season. While the highest peak yield was reached in the cows that calved in winter, the persistency in spring and summer calvers was better than the others. It was observed that the peak yield was affected by age (P<0.001) and increased gradually until 13. Even if the significance wasn't determined, the tendency of least squares means showed there is an opposite trend in the persistency.

Table 1. The least square result of the growth traits and daily gain between those traits of calves different ages and factors.

Factors	BW(kg)		WW(kg)		SMW(kg)		YM(kg)		DGBWW(kg)		DGBSM(kg)		DGBTM(kg)		DGSTM(kg)	
	n	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$
μ	***2330***	***30.43±0.21***	***552***	**97.79±1.48**	***1808***	**112.98±1.13**	***1018***	**169.40±2.21**	***552***	**0.441±0.011**	***1808***	**0.459±0.006**	***1018***	**0.382±0.006**	***1018***	**0.306±0.009**
Year	p	***		NS		***		NS	p	NS		***		*		NS
2016	*525*	30.13±0.27[b]	-	-	*483*	116.55±1.37[a]	*316*	169.84±2.57	-	-	*483*	0.480±0.008[a]	*316*	0.383±0.007[ab]	*316*	0.297±0.011
2017	*551*	29.31±0.27[c]	-	-	*456*	115.51±1.39[a]	*303*	171.57±2.67	-	-	*456*	0.479±0.008[a]	*303*	0.390±0.007[a]	*303*	0.308±0.012
2018	*587*	30.62±0.27[b]	*104*	99.03±2.10	*496*	110.13±1.37[b]	*399*	166.79±2.55	*104*	0.451±0.015	*496*	0.442±0.008[b]	*399*	0.373±0.007[b]	*399*	0.315±0.011
2019	*667*	31.65±0.25[a]	*448*	96.55±1.30	*373*	109.74±1.54[b]	-	-	*448*	0.430±0.009	*373*	0.434±0.009[b]	-	-	-	-
Season	p	NS		*		***		**	p	**		***		**		NS
winter	*249*	30.47±0.33	*71*	100.81±2.23[a]	*220*	119.76±1.60[a]	*102*	177.41±3.12[a]	*71*	0.476±0.016[a]	*220*	0.496±0.009[a]	*102*	0.404±0.009[a]	*102*	0.302±0.013
spring	*1430*	30.72±0.17	*371*	98.29±1.40[ab]	*1222*	113.34±0.94[b]	*707*	168.93±1.62[ab]	*371*	0.432±0.010[b]	*1222*	0.459±0.005[b]	*707*	0.380±0.005[b]	*707*	0.299±0.007
summer	*556*	30.18±0.24	*110*	94.28±2.18[b]	*315*	107.55±1.39[c]	*189*	162.93±2.45[b]	*110*	0.414±0.016[b]	*315*	0.430±0.008[c]	*189*	0.365±0.007[b]	*189*	0.303±0.011
autumn	*95*	30.34±0.49	-	-	*51*	111.27±2.99[bc]	*20*	168.34±6.60[ab]	-	-	*51*	0.450±0.017[bc]	*20*	0.378±0.018[ab]	*20*	0.323±0.028
Sex	p	***		**		***		***	p	NS		***		***		***
female	*1145*	29.62±0.23[b]	*283*	96.00±1.63[b]	*908*	110.09±1.23[b]	*492*	162.24±2.41[b]	*283*	0.434±0.012	*908*	0.448±0.007[b]	*492*	0.365±0.007[b]	*492*	0.287±0.010[b]
male	*1185*	31.24±0.23[a]	*269*	99.58±2.18[a]	*900*	115.87±1.23[a]	*526*	176.57±2.38[a]	*269*	0.446±0.012	*900*	0.470±0.007[a]	*526*	0.399±0.007[a]	*526*	0.326±0.010[a]
Age of dam	p	***		NS		NS		NS	p	NS		NS		NS		*
...< 4	*460*	29.19±0.27[c]	*104*	96.53±2.00	*364*	110.69±1.41	*193*	169.79±2.79	*104*	0.437±0.014	*364*	0.453±0.008	*193*	0.387±0.008	*193*	0.328±0.012[a]
4≤…<7	*849*	30.16±0.23[b]	*177*	98.09±1.63	*673*	112.63±1.21	*385*	167.53±2.34	*177*	0.445±0.012	*673*	0.458±0.007	*385*	0.376±0.006	*385*	0.299±0.010[ab]
7≤…<10	*597*	30.84±0.25[a]	*150*	97.86±1.79	*451*	112.43±1.36	*256*	170.58±2.62	*150*	0.436±0.013	*451*	0.453±0.008	*256*	0.384±0.007	*256*	0.321±0.011[ab]
10≤…<13	*314*	30.64±0.31[ab]	*84*	100.66±2.11	*237*	114.37±1.64	*141*	172.12±3.11	*84*	0.454±0.015	*237*	0.465±0.009	*141*	0.388±0.009	*141*	0.316±0.013[ab]
13≤…<22	*110*	31.31±0.47[a]	*37*	95.81±3.00	*83*	114.79±2.45	*43*	167.00±4.79	*37*	0.431±0.021	*83*	0.464±0.014	*43*	0.374±0.013	*43*	0.269±0.021[b]
Age of weaning weight			p	***					p	NS						
90≤...<135	-	-	*295*	82.66±1.33[c]	-	-	-	-	*295*	0.448±0.010	-	-	-	-	-	-
135≤…<180	-	-	*207*	95.89±1.76[b]	-	-	-	-	*207*	0.432±0.013	-	-	-	-	-	-
180≤…<217	-	-	*50*	114.82±2.72[a]	-	-	-	-	*50*	0.441±0.020	-	-	-	-	-	-

DGBWW; Daily gain between birth weaning. DGBSM; Daily gain between birth and six month. DGSTM; Daily gain between six and twelve month. Village: This factor is highly significant for all traits and has so many subclasses, because of the importance level is not shown in the table; NS: non-significant (P>0.05); *P<0.05; **P<0.001; a, b, c, Differences between groups with different letters in the same column are significant. -: There is no factors affecting the traits.

Table 2. The least square result of the reproduction traits of cows and factors.

Factors	CI(day)		SP(day)	
	n	$x \pm S_{x}$	*n*	$x \pm S_{x}$
µ	***653***	**470.08±9.32**	***653***	**150.08±9.32**
Calving year				
2016	*210*	489.70 ±11.30[a]	*210*	169.10 ±11.30[a]
2017	*234*	482.40±10.40[a]	*234*	162.40±10.40[a]
2018	*209*	438.80±11.00[b]	*209*	158.80±11.00[b]
	p	***		****
Calving season				
winter	*97*	441.30±12.00[b]	*97*	121.30±12.00[b]
spring	*435*	450.32±7.22[b]	*435*	130.32±7.22[b]
summer	*105*	486.70±11.10[a]	*105*	166.70±11.10[a]
autumn	*16*	502.00±25.40[a]	*16*	182.00±25.40[a]
	p	**		**
Age of calving (year)				
...<4	*145*	501.20±10.90[a]	*145*	181.20±10.90[a]
4≤…<7	*233*	467.75±9.78[b]	*233*	147.75±9.78[b]
7≤…<10	*170*	467.30±10.20[b]	*170*	147.30±10.20[b]
10≤…<13	*86*	470.80±13.10[b]	*86*	150.80±13.10[b]
13≤…<20	*19*	443.30±24.00[b]	*19*	123.30±24.00[b]
	p	**		**

Village: This factor is highly significant for all traits and has so many subclasses, because of the importance level is not shown in the table; NS: non-significant (P>0.05); *P<0.05; **P<0.001; [a, b, c,] Differences between groups with different letters in the same column are significant.

Table 3. The least square result of the production traits of cows and factors.

Factors		LMY(kg)		MY/LP(kg)		MY/CI(kg)		DPY(day)		PY(kg)		P(%)	
		N	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$	*n*	$\bar{x}\pm S_{\bar{x}}$
	μ	***923***	**860.40±17.60**	***923***	**4.447±0.095**	***334***	**1.916 ±0.082**	**921**	**83.34±3.34**	**921**	**5.589±0.116**	**921**	**77.35±1.04**
Calving year	2016	*228*	797.10±21.30^{b}	*228*	4.282±0.113^{b}	*110*	1.654±0.100^{b}	*228*	80.88±4.00^{b}	*228*	5.295±0.139^{b}	*228*	77.95±1.24^{b}
	2017	*206*	908.40±22.10^{a}	*206*	4.909±0.119^{a}	*114*	1.831±0.091^{b}	*226*	84.08±4.06^{ab}	*226*	5.904±0.141^{a}	*226*	81.67±1.26^{a}
	2018	*280*	918.20±21.30^{a}	*280*	4.615±0.115^{a}	*110*	2.298±0.103^{a}	*283*	93.57±3.97^{a}	*283*	5.574±0.138^{ab}	*283*	78.32±1.23^{b}
	2019	*209*	817.90±24.30^{b}	*209*	4.103±0.130^{b}	-	-	*184*	74.82±4.66^{b}	*184*	5.584±0.162^{ab}	*184*	71.32±1.45^{c}
		P	***		***		***		***		***		***
Calving season	winter	*129*	926.80±23.60^{a}	*129*	4.640±0.124	*58*	2.342±0.106^{a}	*126*	95.02±4.33^{a}	*126*	5.920±0.150^{a}	*126*	76.27±1.34^{ab}
	spring	*631*	836.70 ±14.40^{b}	*631*	4.549±0.077	*225*	1.942±0.065^{b}	*634*	70.30±2.66^{b}	*634*	5.653±0.093^{ab}	*634*	79.27±0.83^{a}
	summer	*142*	796.90 ±21.20^{b}	*142*	4.424±0.111	*40*	1.611±0.117^{c}	*141*	67.35±3.90^{b}	*141*	5.335±0.136^{b}	*141*	79.68±1.21^{a}
	autumn	*21*	881.20±49.90^{ab}	*21*	4.297±0.270	*11*	1.816±0.218^{bc}	*20*	100.69±9.55^{a}	*20*	5.449±0.332^{ab}	*20*	74.18±2.96^{b}
		P	***		NS		***		***		**		***
Age of calving (year)	...<4	*179*	821.50±22.70^{b}	*179*	4.258±0.123^{b}	*67*	1.743±0.106	*177*	95.41±4.31^{a}	*177*	5.246±0.150^{b}	*177*	78.20±1.34
	4≤…<7	*336*	862.30±19.70^{ab}	*336*	4.453±0.107^{ab}	*121*	1.923±0.090	*334*	84.44±3.72^{b}	*334*	5.621±0.129^{a}	*334*	77.00±1.15
	7≤…<10	*234*	900.90±20.50^{a}	*234*	4.661±0.111^{a}	*88*	1.996±0.094	*233*	81.12±3.90^{b}	*233*	5.834±0.135^{a}	*233*	75.89±1.21
	10≤…<13	*126*	857.30±25.00^{ab}	*126*	4.537±0.135^{ab}	*46*	1.868±0.120	*130*	77.18±4.73^{b}	*130*	5.754±0.164^{a}	*130*	76.04±1.47
	13≤…<20	*48*	860.00±35.30^{ab}	*48*	4.477±0.191^{ab}	*12*	2.108±0.210	*47*	78.54±6.67^{b}	*47*	5.491±0.232^{b}	*47*	79.61±2.07
		P	*		*		NS		**		***		*
Lactation period (day)	100≤...<160	*229*	671.60±21.90^{c}	-	-	-	-	-	-	-	-	-	-
	160≤…<220	*450*	873.40±20.10^{b}	-	-	-	-	-	-	-	-	-	-
	220≤…<366	*244*	1036.30±21.90^{a}	-	-	-	-	-	-	-	-	-	-
		P	***										

Village: This factor is highly significant for all traits and has so many subclasses, because of the importance level is not shown in the table; NS: non-significant (P>0.05); *P<0.05; **P<0.001; $^{a, b, c,}$ Differences between groups with different letters in the same column are significant.

DISCUSSION

While the birth, weaning, and sixth-month weights are similar to the values determined (Shahin et al., 2010; Çelikeloğlu et al., 2015; Iam 2019) in different buffalo breeds, they are slightly ahead of the others (Thiruvenkadan et al. 2009; Akhtar et al. 2012; Uğurlu et al. 2016). Weight at one year was between the ranges (134.20-188.83 kg) reported by some researchers (Thiruvenkadan et al. 2009; Shahin et al. 2010; Akhtar et al. 2012; Çelikeloğlu et al. 2015). Village and sex significantly ($P<0.05$) affected all of these traits. While some researchers (Thiruvenkadan et al. 2009; Akhtar et al. 2012; Uğurlu et al. 2016; Iam 2019) found the effects of year, season, sex, and age of dam on birth weight to be similarly significant, Çelikeloğlu et al (2015) stated that the effect of age of dam was not significant. Akhtar et al (2012) showed that the effect of the year of birth, season, and age of dam was significant on WW in Nili Ravi. This was compatible with the present study. The significant effect of sex and dam age on sixth-month weight in Anatolian buffaloes notified Çelikeloğlu et al (2015) was consistent with the present study, but the result of Thiruvenkadan et al. in Murrah buffaloes was different. In one-year weight, our findings were similar to the consequence of significant seasonal effects in Nili Ravi by Akhtar et al. But the effects of birth season in Murrah, and sex in Anatolian buffaloes were not found to be similar (Thiruvenkadan et al. 2009; Çelikeloğlu et al. 2015). Differences may be due to breed, husbandry, climate, care and feeding. It was determined that the effect of village and birth season on DGBWW was significant ($P<0.01$) in Egypt and Nili Ravi buffaloes (Shahin et al. 2010; Akhtar et al. 2012). While the findings of our study were consistent with the report of Akhtar et al (2012) on the birth season, contradicted in year and dam age. The effects of village, season, year, and sex on DGBSM and DGBTM traits are significant ($P<0.05$; $P<0.001$). This situation is different from the nonsignificant determination for the effect of year and season by Shahjahan et al (2017). The CI is between 385 and 560 days reported in Anatolian and Murrah buffaloes (Tekerli et al. 2001; Küçükkebapçı and Aslan 2002; Şekerden 2013; Dev et al. 2016; Soysal et al. 2018; Patil et al. 2018; Koçak et al. 2019; Alkoyak and Öz 2020). These researchers stated that the long CI may be due to lactation stress especially in high-yielding buffaloes, and the seasonality of reproduction. While the SP is slightly below the values reported by different researchers (Cady et al. 1983; Mostafa et al. 2017; Patil et al. 2018) in Murrah, Nili Ravi, and Egyptian buffaloes, it is among the values reported in Anatolian buffaloes (Küçükkebapçı and Aslan 2002). It was determined that these two reproductive traits were significantly ($P<0.01$) affected by all of the factors. This finding is compatible with the significant determination of the region, year, season and age effect detected in Anatolian, Murrah and Nili Ravi (Cady et al. 1983; Tekerli et al. 2001; Dev et al. 2016; Patil et al. 2018; Soysal et al. 2018; Koçak et al. 2019; Alkoyak and Öz 2020). LMY is between 894-1223 kg reported in Anatolian buffaloes (Tekerli et al. 2001; Borghese 2005; Tekerli 2016; Soysal et al. 2018; Koçak et al. 2019; Alkoyak and Öz 2020). However, it is slightly below the values reported in Nili Ravi, Murrah and Egyptian buffaloes (Bashir et al. 2015; Pandey et al. 2015; Sigdel et al. 2015; Dev et al. 2016; Mostafa et al. 2017; Patil et al. 2018; Iam 2019). This may be due to differences in breed, geographical and breeding conditions. The lack of controlled selection before the breeding project may have been effective in this fact. Lactation milk yield was significantly ($P<0.05$) affected by village, calving year, season, age and lactation period. This finding is consistent with the results of Cady et al (1983) and Bashir et al (2015). Different researchers (Soysal et al. 2018; Koçak et al. 2019; Akolyak and Öz 2020) found the effect of calving year, season and age to be significant, similar to this study. MY/LP is just below 5.08 kg per buffalo reported by Borghese in Anatolian buffaloes. In addition, this finding is behind reports (Sigdel et al. 2015; Dev et al. 2016; Patil et al. 2018) in Murrah buffaloes. MY/LP was significantly ($P<0.05$) affected by the village, calving year and age. This finding is consistent with the significant determination of the effect of year, season and age in Murrahs (Sigdel et al. 2015; Dev et al. 2016; Patil et al. 2018). MY/CI is below the values found in Murrahs (Jakhar et al. 2017; Patil et al. 2018). This trait was significantly ($P<0.05$) affected by the village, calving year and season. This finding is harmonious with the reports of Jakhar et al. and Patil et al in Murrah buffaloes. DPY is longer than determined (Tekerli et al. 2001; Thiruvenkadan 2011; Galsar et al. 2016) in Anatolian, Murrah and Mehsana buffaloes. DPY is affected by all factors with a moderate significance ($P<0.01$). This finding is consistent with the report of the significant period and season effect in Anatolian and Murrahs (Tekerli et al. 2001; Thiruvenkadan 2011). PY is lower than stated in different buffalo breeds (Tekerli et al. 2001; Thiruvenkadan 2011; Dev et al. 2016; Galsar et al. 2016; Patil et al. 2018). PY is significantly ($P<0.05$) affected by all of the factors. This finding is consistent with Tekerli et al (2001), Thiruvenkadan (2011), and Dev et al (2016) in terms of period effect. Persistence was behind the reports (Chaudhry et al. 2000; Mostafa et al. 2017) in Nili Ravi and Bulgarian Murrah, and ahead of Anatolian and Egyptian buffaloes (Tekerli et al. 2001; Elmaghraby 2010). The method of calculating the persistence and the number of data may have caused the difference. This trait was significantly ($P<0.05$) affected by the village, calving year, and season. This finding is consistent with the

reports of different researchers (Chaudhry et al. 2000; Penchev and Peeva 2013) in terms of year, period and seasonal effects.

CONCLUSION

As a conclusion, since the examined traits are affected by environmental factors, care and management should be arranged accordingly and this should be taken into account in the selection of breeder animals. The environmental factors should be considered for stable production. The seasonal breeding tendency should be considered and investigated with the aspects of the reasons. The increase in demand of customers in winter months should not be ignored from an economic point of view. It has been concluded that the milk yield increases until the age of 7 to 10 years in the buffaloes in Yozgat, and the performance decreases after the age of 13, so it is not beneficial to keep the older buffalo cows.

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RESEARCH ARTICLE

Novel Thiazole/Ethyl Thiazole Carboxylate-Acetamide Derivatives and Their Cytotoxic Effect Evaluation

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ABSTRACT

In this study, the main goal is to determine the anticancer compound(s) that can be used against A549 non-small lung epithelial carcinoma, Caco-2 colon carcinoma, and SHSY-5Y neuroblastoma cells with high selectivity. For this purpose, our study group synthesized two similar acetamide series: four compounds (3a–3d), including thiazole, and four compounds (3e–3h), including ethyl (4-methyl-thiazol-5-yl)carboxylate. The structural analyses of eight compounds were identified by HRMS, ^{1}H-NMR, and ^{13}C-NMR. After approving the purity, their anticancer profiles were evaluated against above cancer cells, and the cytotoxicity effect was also tested against NIH/3T3 fibroblast cells. Meanwhile, ADME and DFT calculations indicated that compounds have good ADME profiles and chemical stability. Among the targeted compounds, compound 3g exhibits greater stability. In chemical systems, stability is important because it represents the energy balance within a molecule. The results showed that compounds have significant impact on SHSY-5Y cells with higher selectivity than other cells. The combination of ester groups on thiazole and thiazoline (compound 3g) was found to be significantly more effective than doxorubicin and highly selective on SHSY-5Y cells than healthy cells. Besides that, combination of thiazole and triazole (3d and 3h) decreased antiproliferative activity in three cancer cells while increasing cytotoxicity in healthy cells. This study suggests that future perspectives in studies regarding the treatments of neuroblastoma and its related diseases of ethyl 2-acetamido-4-methylthiazole-5-carboxylate and thiazoline combination are encouraging.

Keywords: Anticancer activity, DFT, Ethyl carboxylate, SHSY-5Y, Thiazole

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Yeni Tiyazol/Etil Tiyazol Karboksilat-Asetamid Türevleri ve Bunların Sitotoksik Etkisinin Değerlendirilmesi

ÖZ

Bu çalışmada A549 küçük olmayan akciğer epitelyal karsinomu, Caco-2 kolon karsinomu ve SHSY-5Y nöroblastoma hücrelerine karşı kullanılabilecek yüksek seçiciliğe sahip antikanser bileşik(ler)in belirlenmesi temel amaçtır. Bu amaçla çalışma grubumuz tarafından tiyazol içeren dört bileşik (**3a-3d**) ve etil (4-metil-tiyazol-5-il)karboksilat içeren dört bileşik (**3e-3h**) şeklinde benzer iki asetamit serisi sentezlendi. Bu sekiz bileşiğin yapısal analizleri HRMS, ^{1}H-NMR ve ^{13}C-NMR yöntemleri ile gerçekleştirildi. Bileşiklerin saf bir şekilde elde edildikleri tespit edildikten sonra bahsedilen kanser hücrelerine karşı antikanser profilleri değerlendirildi ve ayrıca NIH/3T3 fibroblast hücrelerine karşı sitotoksik etkisi incelendi. Aynı zamanda ADME ve DFT hesaplamaları sonucunda bileşiklerin iyi ADME profiline ve kimyasal stabiliteye sahip olduğu belirlendi. Hedeflenen bileşikler arasında bileşik **3g** daha fazla stabilite sergilemektedir. Kimyasal sistemlerde stabilite önemlidir çünkü bir molekül içindeki enerji dengesini temsil eder. Sonuçlar, bileşiklerin SHSY-5Y hücreleri üzerinde diğer hücrelere göre daha seçici bir etkiye sahip olduğunu gösterdi. Tiyazol üzerindeki ester grubu ile tiyazolidin (bileşik **3g**) kombinasyonunun doksorubisinden anlamlı derecede etkili olduğu ve SHSY-5Y hücreleri üzerinde sağlıklı hücrelere göre oldukça seçici olduğu bulundu. Bunun yanı sıra, tiyazol-triazol kombinasyonu (**3d** ve **3h**) üç kanser hücresinde de antiproliferatif aktiviteyi azaltırken, sağlıklı hücrede sitotoksisiteyi arttırdı. Bu çalışma ile nöroblastoma ve bununla ilişkili hastalıkların tedavisine ilişkin çalışmalarda etil 2-asetamido-4-metiltiyazol-5-karboksilat ve tiyazolin kombinasyonunun ileride gerçekleştirilecek çalışmalar için ümit verici olduğunu ileri sürmektedir.

Anahtar Kelimeler: Antikanser aktivite, DFT, Etil karboksilat, SHSY-5Y, Tiyazol

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INTRODUCTION

Cancer is a general term used to describe a number of diseases that are caused by the uncontrolled division of cells. (Hernandes et al. 2023). Regardless of the type of cancer, necrotic death of cancer cells is one of the main problems (Ocansey et al. 2024; Sharifi et al. 2019). Even worse, it does not affect only humankind but also affects animals. Human and animal cancer mortality rates show that the most common cause of death is (Oh & Cho 2023a; Sarver et al. 2022). Moreover, in decades, the researchers explored the similarity between the prognosis of cancer in humans and animals (Cavalier et al. 2023; Cortes 2019), as well as the helpful and unhelpful aspects of having a companion animal for people with cancer who are dealing with the emotional challenges that come with diagnosis and treatment. (McGhee et al. 2022; Nitkin 2014; Nitkin & Buchanan 2020). Although these reports were tested on a small-scale population part by part, they all indicated very similar results. The pathophysiological development of cancer in humans and animals is very similar, and the treatment of cancer in both species together has a positive impact on treatment. This means that the treatment options can be applied to both humans and their pets (Oh & Cho 2023b; Pinho et al. 2012).

Approaches in cancer treatments vary widely, especially radiotherapy (Delaney et al. 2005; Freitas et al. 2023), chemotherapy (Falzone et al. 2023; Xing et al. 2024; Yang et al. 2023), and biomarkers (Dora et al. 2023; Tarighati et al. 2023) are promising in many ways. In the last two decades, there have been valuable improvements after the approval of some small-molecule inhibitors (Gallego & Varani 2001; Hoelder et al. 2012; Roskoski Jr 2024; Wu et al. 2015). Unfortunately, the incidence of cancer is increasing day by day and, in many cases, the misdiagnosis or the incorrect application of the treatment can worsen the condition of the patient. (Kavitha et al. 2022; Kwon et al. 2015). For this reason, the protocols for the treatment of cancer should be reorganized and improved. Besides, the experience clinically in the past points out that the other real problem in the future will be useless because of developing resistance against current drugs (Eslami et al. 2024; Holohan et al. 2013; Housman et al. 2014; Li et al. 2024; Tolomeo & Simoni 2002). Because of that, cancer studies must go on immediately in every aspect. For example, designing and synthesizing new chemotherapeutics seems like a useful option to address this issue (Laiolo et al. 2024; Xiong et al. 2024). Since we perpetually need a new agent that has a selective anticancer effect, in this study, we designed and synthesized novel nitrogen-containing heterocyclic molecules to test their antitumoral activity. The main core was formed with two different thiazole rings, and their derivatives were designed with imidazole, triazole, tetrazole, and thiazoline rings linked with an acetamide bridge. Here, one of the main purposes is to discuss the effect of the ester group of thiazole, and the second is to determine the anticancer activity ability of the heterocycles. We chose these varieties because the cytotoxic activity of thiazole (Evren et al. 2023; Özkay et al. 2022), imidazole (Osmaniye et al. 2022), triazole (Saffour et al. 2024), tetrazole (Dileep et al. 2017) and thiazoline (Altintop et al. 2014) was previously reported against various cancer cells. Additionally, the drugs approved for anticancer treatments, as shown in Figure 1, include these ring systems.

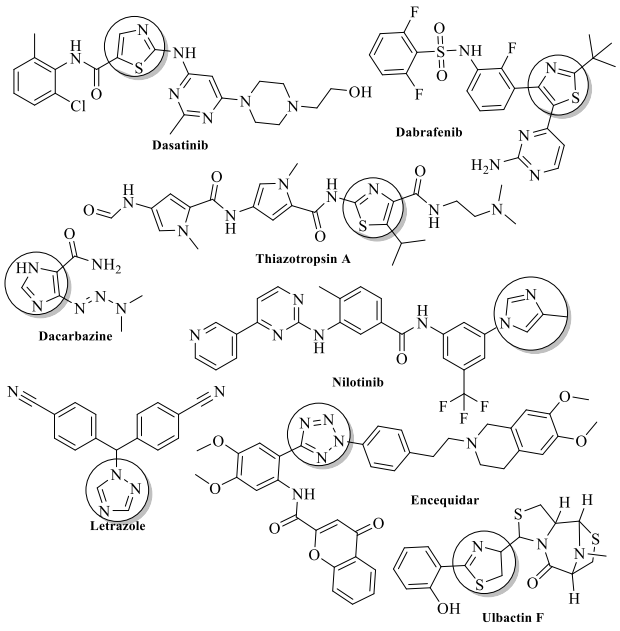


Figure 1: Some anticancer drugs and their ring systems.

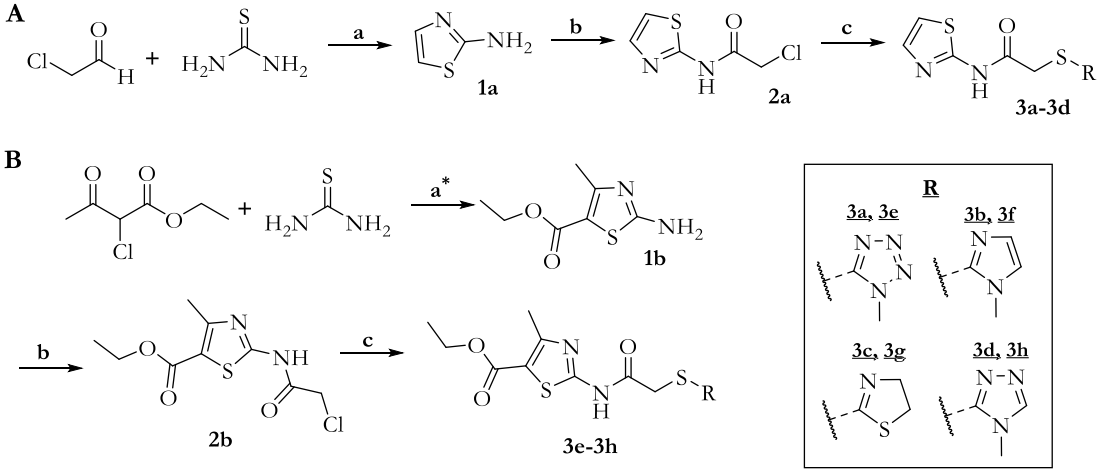
In addition to the above, thiazole ring was reported many times because of its anticancer properties against A549, Caco-2, and SH-SY5Y cell lines. Against A549 cells, when this ring system was especially linked with amide bridge (Evren et al. 2019a; Evren et al. 2019b) showed valuable cytotoxicity effects. On the other hand, according to the literature (Bhagat et al. 2021; Catarzi et al. 2022; El-Gazzar et al. 2017; Singh et al. 2022), thioether group linked to thiazole positively affected anti-neuroblastoma and anti-colon adenocarcinoma activities. Also, the thiazole ring system is favorable in anticancer drug development due to inducing apoptosis properties by medicinal chemists (Ahmed et al. 2009; Bhat et al. 2009; Wang et al. 2024; Xiong et al. 2019). All these properties inspired us to develop new anticancer molecules using thiazole core combined with thioether and amide bridges. Therefore, it was portended that using this combination will successfully result in reaching novel and effective anticancer agents.

Given the foregoing details, two related series (thiazole-acetamide and novel ethyl thiazole carboxylate-acetamide analogs) were generated and synthesized for this study, after which their cytotoxicity and physicochemical profiles were examined. Finally, the structure-activity relationship (SAR) was reported.

MATERIALS AND METHODS

Chemistry

All chemicals used in the syntheses were purchased either from Merck Chemicals (Merck KGaA, Darmstadt, Germany) or Sigma-Aldrich Chemicals (Sigma-Aldrich Corp., St. Louis, MO, USA). The compounds were monitored for reactions and purities using thin-layer chromatography (TLC) with silica gel 60 F254 aluminum sheets that were purchased from Merck (Darmstadt, Germany). The MP90 digital melting point apparatus (Mettler Toledo, Ohio, USA) was used to record the melting points of the synthesized compounds, and the results were given without correction. ^{1}H NMR and ^{13}C NMR spectra were recorded by a Bruker 300 MHz and 75 MHz digital FT-NMR spectrometer (Bruker Bioscience, Billerica, MA, USA) in DMSO-d_6, respectively. Splitting patterns in the NMR spectra were denoted by the following symbols: s for singlet, d for doublet, t for triplet, and m for multiplet. Coupling constants (J) were reported as Hertz. High-resolution mass spectrometric (HRMS) analyses were performed using an LC/MS-IT-TOF system (Shimadzu, Kyoto, Japan). Elemental analyses were performed on a Leco 932 CHNS analyzer (Leco, Michigan, USA). The whole synthesis plan is illustrated in Scheme 1.



Scheme 1. Synthesis plan. A: Stepwise synthesis of compounds 3a-3d, B: Stepwise synthesis of compounds 3e-3h; a: EtOH, reflux 4 hrs; **a*:** THF, 0°C/rt 30 min/reflux 8 hrs; **b:** 2-chloroacetyl chloride, THF, TEA, 0°C, **c:** Mercaptoheterocycles, Me_2CO, K_2CO_3, rt. 2 hrs.

Synthesis of 2-aminothiazole and ethyl 2-amino-5-methylthiazole-4-carboxylate (1a, 1b)

Chloro acetaldehyde (40.76 mmol, 2.59 ml) was mixed with ethanol and added slowly into a flask containing an equivalent amount of thiourea (40.76 mmol, 3.103 g) in ethanol while in an ice bath. The mixture was stirred at room temperature for 4 hours. The mixture was then poured into ice-cold water. The resulting precipitate was filtered and, after drying, recrystallized in ethanol.

Ethyl 2-chloroacetoacetate (18.84 mmol, 2.61 ml) was added dropwise into a flask containing thiourea (18.84 mmol, 1.434 g) dissolved in tetrahydrofuran in a cold environment controlled using an ice bath. After the addition of the acetate derivative, the mixture was stirred for 30 minutes in the same conditions, then refluxed for 8 hours. Following the end of the reaction, the solvent was evaporated, and the residue was recrystallized from ethanol.

Synthesis of 2-chloro-N-(thiazol-2-yl)acetamide (2a) / ethyl 2-(2-chloroacetamido)-5-methylthiazole-4-carboxylate (2b)

To the previously obtained compound 1a/1b dissolved in tetrahydrofuran (THF), triethyl amine (TEA) (1: 3 eq.) was added, followed by the dropwise addition of 2-chloroacetyl chloride (1: 1 eq.), while the reaction conditions were controlled by an ice bath. The mixture was stirred for another hour after the addition. After determining the end of the reaction using TLC, THF was evaporated, and the remaining residue was washed using water and filtered. The final residue was recrystallized from ethanol.

Synthesis of 2-chloro-N-(thiazol-2-yl) acetamide derivatives (3a-3d) and ethyl 2-(2-chloroacetamido)-5-methylthiazole-4-carboxylate (3e-3h)

Compound 2a/2b was added to a solution of the appropriate mercapto derivative (1 eq.) and potassium carbonate (1.5 eq.) in acetone. 1-Methyl-1H-tetrazole-5-thiol, 1-methyl-1H-imidazole-2-thiol, 4,5-dihydrothiazole-2-thiol, and 4-methyl-4H-1,2,4-triazole-3-thiol were the mercapto derivatives used in the synthesis, as illustrated in Scheme 1. The mixture was stirred at room temperature for 2 minutes, and its completeness was checked using TLC. Following the evaporation of the solvent, the residue was cleaned and collected through filtration. The final residue was recrystallized from ethanol.

Ethyl 5-methyl-2-[2-((1-methyl-1H-tetrazol-5-yl) thio)acetamido]thiazole-4-carboxylate (3e)

m. p. 206-207 °C, ^{1}H NMR (300 MHz) (DMSO-d_6) δ (ppm): 1.27 (t, J= 7.08 Hz, 3H, aliphatic CH_3), 2.54 (s, 3H, thiazole- CH_3), 3.98 (s, 3H, tetrazole- CH_3), 4.22 (q, J= 7.10 Hz, 2H, aliphatic CH_2), 4.34 (s, 2H, CO-CH_2-S), 12.85 (brs, H, N-H). ^{13}C NMR (75 MHz) (DMSO-d_6) δ (ppm): 14.67 (thiazole- CH_3), 17.52 (aliphatic- CH_3), 34.14 (tetrazole- CH_3), 37.20 (CO-CH_2-S), 60.88 (COO-CH_2-), 114.24 (thiazole C-5), 153.64 (thiazole C-4), 156.76 (tetrazole C-5), 161.03 (thiazole C-2), 162.57 (carboxyl), 167.50 (carbonyl). For $C_{11}H_{14}N_6O_3S_2$ calculated: Elem. Anal.: C, 38.59%; H, 4.12%; N, 24.54%; O, 14.02%; S, 18.73%, found: C, 38.56%; H, 4.11%; N, 24.57%; O, 14.02; S, 18.74%. HRMS (m/z): $[M + 1]^+$ calculated 343.0642; found 343.0642.

Ethyl 5-methyl-2-[2-((1-methyl-1H-imidazol-2-yl)thio)acetamido]thiazole-4-carboxylate (3f)

m. p. 275-276 °C, ^{1}H NMR (300 MHz) (DMSO-d_6) *δ* (ppm): 1.27 (t, *J*= 7.09 Hz, 3H, aliphatic CH_3), 2.54 (s, 3H, thiazole- CH_3), 3.59 (s, 3H, imidazole- CH_3), 3.97 (s, 2H, CO-CH_2-S), 4.23 (q, *J*= 7.09 Hz, 2H, aliphatic CH_2), 6.94 (d, *J*= 1.09 Hz, H, imidazole-H), 7.26 (d, *J*= 1.01 Hz, H, imidazole-H), 12.83 (brs, H, N-H). ^{13}C NMR (75 MHz) (DMSO-d_6) *δ* (ppm): 14.65 (thiazole-CH_3), 17.48 (aliphatic- CH_3), 33.46 (imidazole- CH_3), 37.38 (CO-CH_2-S), 61.01 (COO-CH_2-), 114.67 (thiazole C-5), 124.19 (imidazole C-5), 129.12 (imidazole C-4), 135.10 (imidazole C-2), 156.72 (thiazole C-4), 159.80 (thiazole C-2), 162.50 (carboxyl), 168.35 (carbonyl). For $C_{13}H_{16}N_4O_3S_2$ calculated: Elem. Anal.: C, 45.87%; H, 4.74%; N, 16.46%; O, 14.10%; S, 18.84%, found: C, 45.85%; H, 4.71%; N, 16.43%; O, 14.12%; S, 18.86%. HRMS (m/z): $[M + 1]^+$ calculated 341.0737; found 341.0744.

Ethyl 2-[2-((4,5-dihydrothiazol-2-yl)thio)acetamido]-5-methylthiazole-4-carboxylate (3g)

m. p. 275-276 °C, ^{1}H NMR (300 MHz) (DMSO-d_6) *δ* (ppm): 1.27 (t, *J*= 7.09 Hz, 3H, aliphatic CH_3), 2.54 (s, 3H, thiazole-CH_3), 3.47 (t, *J*= 7.99 Hz, 2H, dihydrothiazole-H), 4.09 (t, *J*= 7.99 Hz, 2H, dihydrothiazole-H), 4.18 (s, 2H, CO-CH_2-S) 4.24 (q, *J*= 7.09 Hz, 2H, aliphatic-CH_2), 12.70 (brs, H, N-H). ^{13}C NMR (75 MHz) (DMSO-d_6) *δ* (ppm): 14.65 (thiazole-CH_3), 17.47 (aliphatic-CH_3), 36.04 (CO-CH_2-S), 36.20 (dihydrothiazole C-5), 61.00 (COO-CH_2-), 64.35 (dihydrothiazole C-4) 114.67 (thiazole C-5), 156.68 (thiazole C-4), 159.84 (thiazole C-2), 162.49 (dihydrothiazole C-2), 162.86 (carboxyl), 167.35 (carbonyl). For $C_{12}H_{15}N_3O_3S_3$ calculated: Elem. Anal.: C, 41.72%; H, 4.38%; N, 12.16%; O, 13.89%; S, 27.85%, found: C, 41.75%; H, 4.36%; N, 12.14%; O, 13.89; S, 27.86%. HRMS (m/z): $[M + 1]^+$ calculated 346.0348; found 346.0352.

Chemical Theoretical Calculations

Theoretical approaches for 2-mercapto-*N*-(thiazol-2-yl)acetamide derivatives (3a-3h) were run using the molecular visualization programs Gaussian 09 W (Frisch et al. 2009) and GaussView 5.0 (Dennington et al. 2009). Density Functional Theory (DFT) calculations were done according to previous studies (Nuha et al. 2022; Nuha et al. 2023). Total electric dipole moment (μ_{tot}) calculated theoretically by using the following equations: (Hernández-Paredes et al. 2009; Kleinman 1962).

$$\mu_{tot} = (\mu_x^2 + \mu_y^2 + \mu_z^2)^{1/2} (1)$$

ADME calculation

The physicochemical descriptors of the final compounds were computed using SwissADME software. Molecular weight (MW), H-bond acceptors number (NHA), H-bond donors number (NHD), topological polar surface area (TPSA), partition coefficient (Log P), and gastrointestinal absorption properties (GI abs) were calculated for compounds 3a–3h.

Biological Activities

Cell line and Cell culture

A549 human lung adenocarcinoma cells (ATCC number CCL-185™), Caco-2 Human Colorectal Adenocarcinoma cells (ATCC number HTB-37™), SH-SY5Y human neuroblastoma cells (ATCC number CRL-2266™) and NIH3T3 mouse healthy fibroblast cells (ATCC number CRL-1658™) were obtained from the American Type Culture Collection. All cells were grown and prepared as descripted in previous studies (Dawbaa et al. 2023; Yurttas et al. 2024; Yurttaş et al. 2023). The control group (solvent control) was prepared with a medium containing 0.1% DMSO. Doxorubicin used as a positive control.

MTT Cytotoxicity assay

The synthesized compounds (3a–3h) were tested for their cytotoxicity in vitro, in comparison with doxorubicin as a reference drug, against A549, Caco-2, SH-SY5Y, and NIH3T3 cells. This method was applied as in previous studies (Dawbaa et al. 2021; Yurttaş et al. 2020; Yurttaş et al. 2019).

RESULTS

Chemistry

The complete synthetic plan of eight targeted compounds (3a–3h) is illustrated in Scheme 1. To synthesize these products, they were divided into two groups as the starting reactants were different. Synthesis of compounds 3a-3d group started with reacting 2-chloroacetaldehyde with equivalent amount of thiourea to produce 2-aminothiazole, which was acetylated with 2-chloroacetyl chloride, and finally the resulting 2-chloro-*N*-(thiazol-2-yl)acetamide was reacted with four different thiol derivatives to produce compounds 3a-3d. The second group, 3e-3h, was synthesized starting with the reaction of ethyl-2-chloroacetoacetate with thiourea. The resulting 2-amino-5-methylthiazole-4-carboxylate was also acetylated using 2-chloroacetychloride, which then reacted with thiol derivatives to produce compounds 3e–3h. Structure elucidation was achieved by ^{1}H-NMR, ^{13}C-NMR, elemental analysis, and High-Resolution Mass Spectrometry (HRMS). The peaks in ^{1}H-NMR and ^{13}C-NMR spectra were observed in the predicted chemical shifts. Elemental analysis and mass peaks [M+1] of the compounds agreed with the predicted molecular formulae.

The ^{1}H-NMR spectra showed singlet peaks at δ 3.98 ppm for methyl hydrogens of tetrazole, while those at 3.59 ppm are for methyl hydrogens of imidazole and triazole. The singlet peak occurring at 2.53-2.54 ppm is for the 5-methyl hydrogens of thiazole. The methylene group bridging the carbonyl with the sulphur atom kept showing between 3.97-4.36 ppm as a singlet. Hydrogen peaks of methylenes numbers 5 and 4 of 4,5-dihydrothiazole belonging to compound 3c are shown as triplets at 3.47 ppm and 4.10 ppm, respectively. In the aromatic region, hydrogens at carbons 5 and 4 of thiazole of compounds 3a–3d kept showing as doublets at around 7.23 ppm and 7.47 ppm, respectively. Hydrogens on carbons 4 and 5 of imidazole in compounds 3b and 3f have doublets at around 6.95 ppm and 7.26 ppm, respectively. A singlet at 8.55 ppm confirms that compound 3d and compound 3h contain only one hydrogen of triazole. The amidic hydrogen present in all synthesized molecules is shown as a broad singlet above 12 ppm.

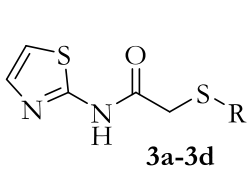
^{13}C-NMR spectra confirmed the ^{1}H-NMR results. The carbon of methyl groups appeared in slightly different shifts according to their position in the molecule. Signals at 34.18, 33.45, and 31.29 ppm were observed to represent the carbon of the methyl group in tetrazole, imidazole, and triazole, respectively. The methyl group carbon in thiazole of compounds 3a-3d showed signals at around 14.65 ppm. The methylene bridge between carbonyl and sulphur showed signals in the range 36-37.5 ppm. The terminal methyl group carbon in the ester of compounds 3e-3h was observed in negligibly different shifts in the range 17.47-17.55 ppm. Similarly, the methylene carbon of the ester kept showing at 60.83-61.01 ppm. The aromatic region in the range of 100-170 ppm showed different signals representing different carbons in the aromatic rings. These carbons were assigned individually in the ^{13}C-NMR analytical monographs below. The carbonyl carbons have shown signals in the range 165.88-168.35 ppm, whereas the signals of the carboxylic carbon of compounds 3e-3h were observed in 161.30-162.86 ppm.

For every targeted compound, the elemental analyses of C, H, and N yielded results that were in line with Mass analyses. It also revealed that the M+1 peaks identified by LC-MS/MS validated the structures of the corresponding molecules and matched their calculated values.

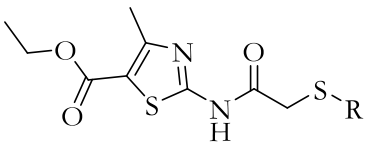
Prediction of physicochemical properties

The pharmacokinetic properties of final compounds were predicted using Swiss ADME and the calculated values were represented in Table 1.

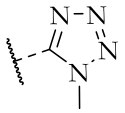
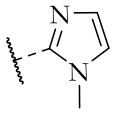
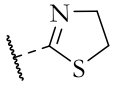
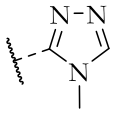
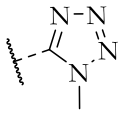
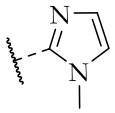
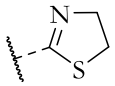
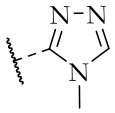
Table 1. Some physicochemical properties of the compounds **(3a-3h)**



3a-3d



3e-3h

Compounds	R	MW	NHA	NHD	TPSA (A°)	Log P	GI abs
3a		256.31	5	1	139.13	0.57	High
3b		254.33	3	1	113.35	1.10	High
3c		259.37	3	1	133.19	1.43	High
3d		255.32	4	1	126.24	0.71	High
3e		342.40	7	1	165.43	1.30	Low
3f		340.42	5	1	139.65	1.82	High
3g		345.46	5	1	159.49	2.17	Low
3h		341.41	6	1	152.54	1.44	Low

MW: Molecular weight, **NHA:** No H-bond acceptors, **NHD:** No H-bond donors, **TPSA:** Topological polar surface area, **Log P:** Partition coefficient, **GI abs:** Gastrointestinal absorption

Molecular weight (MW), number of H-bond acceptors (NHA), number of H-bond donors (NHD), topological polar surface area (TPSA), partition coefficient (Log P), and gastrointestinal absorption properties (GI abs) are some of the important parameters for determining the absorption, distribution, metabolism, and excretion (ADME) processes of a drug in the process leading to a biological response in an organism. According to calculations, compounds 3a–3h possess a molecular weight between 256.31-345.46 g/mol, hydrogen bond acceptor bonds between 3-7 and one hydrogen donor bond. It was seen that there were two more hydrogen acceptors in 3e-3h compounds compared to corresponding 3a-3d compounds due to -COOEt function. The TPSA of the compounds was predicted between 113.35-165.43 Å, whereas log P was calculated between 0.57-2.17. These findings were appropriate to Lipinski rule of five for oral drug probability. Gastrointestinal absorption was predicted as high except three compounds 3e, 3g and 3h.

Theoretical calculations

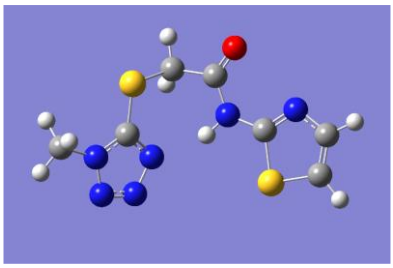
Using DFT/ B3LYP/6-31G(d,p), optimized molecular structures with total energy values of 2-mercapto-*N*-(thiazol-2-yl)acetamide analogs (3a-3h) compounds are shown in Figure 2.

The calculated total energy values for the synthesized molecules obey the following order: 3g < 3e < 3h < 3f < 3c < 3a < 3d < 3b. The final molecules with lower total energy rate indicated that they have a more stable structure, according to computed values of total energy of molecular structures.

The dipole moment of a molecule is a measure of the polarity of the molecule. The value of the dipole moment was also calculated at the DFT/B3LYP/6-

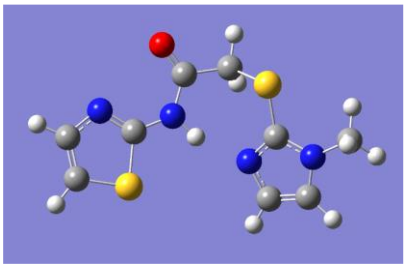
31G(d,p) level using Eqs. (1) and the results are shown in Table 2. Molecule 3f's dipole moment indicates that it is relatively more polarized, while molecule 3c is the less.

In modeling studies where the groups generated from different substituents are used, values of HOMO-LUMO energy, recognized as frontier molecular orbital (FMO) energies, play a pivotal role in determining certain reactivity parameters of the structures.



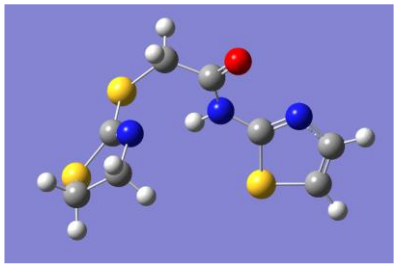
3a

Total Energy: -1471.63175897 a.u.



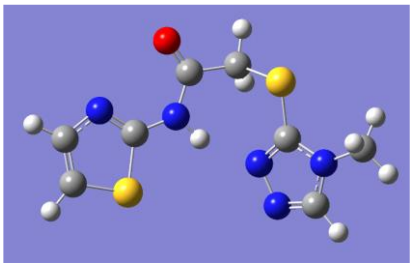
3b

Total Energy: -1439.59825672 a.u.



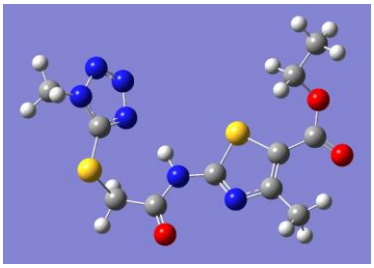
3c

Total Energy: -1744.31660954 a.u.



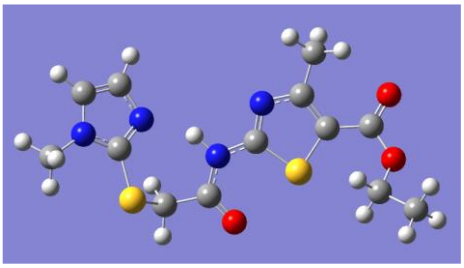
3d

Total Energy: -1455.62007507 a.u.



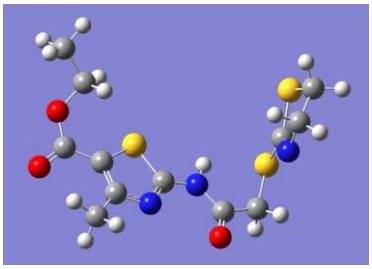
3e

Total Energy: -1778.14166688 a.u.



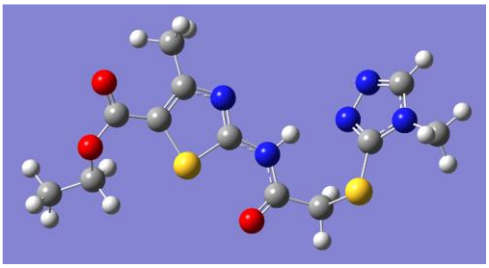
3f

Total Energy: -1746.12065531 a.u.



3g

Total Energy: -2050.82615964 a.u.



3h

Total Energy: -1762.14140196 a.u.

Figure 2: Optimized molecular structures and total energy values of the compounds **3a-3h** by DFT/B3LYP/6-31G(d,p).

Table 2. The values of electric dipole moment of the compounds 3a-3h.

Compounds	μ_x (Debye)	μ_y (Debye)	μ_z (Debye)	μ_{tot} (Debye)
3a	-4.7926	-0.2548	0.9279	4.8882
3b	-6.4479	-3.8892	0.1782	7.5321
3c	-2.2073	-3.0737	0.6205	3.8347
3d	6.9717	-2.3589	1.1115	7.4434
3e	-8.6135	2.7077	2.4430	9.3537
3f	-9.9239	-1.0354	2.3706	10.2555
3g	5.1377	4.6014	0.6106	6.9240
3h	9.3073	-2.4360	0.5746	9.6379

For the 3a-3h compounds, Table 3 indicates that compound 3e has the smallest energy gap (ΔE) with a value of 0.1664 eV, while compound 3c has the biggest energy gap (E) with a value of 0.1948 eV, indicating that compound 3e is more reactive and so, it's less stable among its analogs.

Table 3. Some reactivity parameters of the compounds 3a-3h.

Compounds	E_{HOMO} (eV)	E_{LUMO} (eV)	ΔE (eV)	I (eV)	A (eV)	χ (eV)	η (eV)	S (eV^{-1})	μ (eV)	ω (eV)
3a	-0.2195	-0.0419	0.1776	0.2195	0.0419	0.1307	0.0888	5.6306	-0.1307	0.0962
3b	-0.2099	-0.0261	0.1838	0.2099	0.0261	0.1180	0.0919	5.4407	-0.1180	0.0758
3c	-0.2259	-0.0311	0.1948	0.2259	0.0311	0.1285	0.0974	5.1335	-0.1285	0.0848
3d	-0.2118	-0.0320	0.1798	0.2118	0.0320	0.1219	0.0899	5.5617	-0.1219	0.0826
3e	-0.2253	-0.0589	0.1664	0.2253	0.0589	0.1421	0.0832	6.0096	-0.1421	0.1213
3f	-0.2164	-0.0481	0.1683	0.2164	0.0481	0.1322	0.0841	5.9453	-0.1322	0.1039
3g	-0.2303	-0.0575	0.1728	0.2303	0.0575	0.1439	0.0864	5.7870	-0.1439	0.1198
3h	-0.2189	-0.0523	0.1666	0.2189	0.0523	0.1356	0.0833	6.0024	-0.1356	0.1104

In Figure 3, HOMO-LUMO orbital diagrams of 3e and 3c compounds were showed, whereas others were showed in Figure S24 (see Supplementary Material).

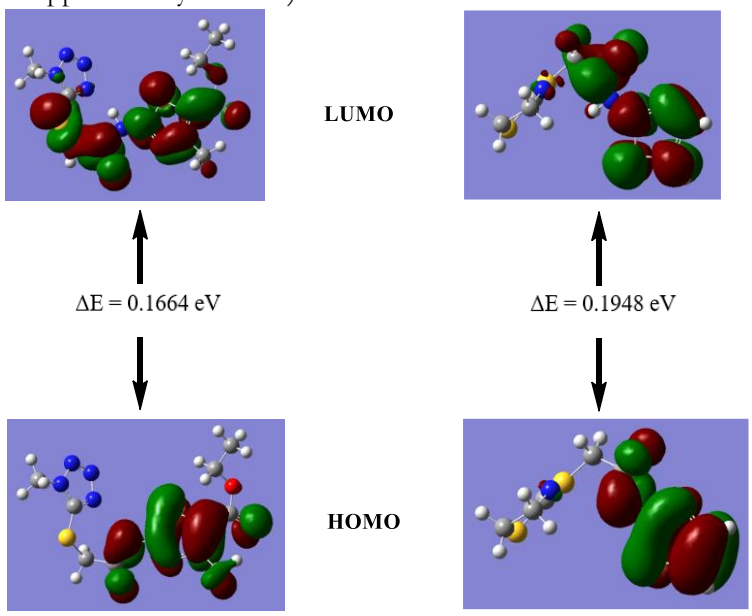


Figure 3: HOMO-LUMO diagrams of the compounds **3e** and **3c** (TD-DFT/B3LYP/6-31G(d,p).

Regarding the compounds in question, 3b exhibits a low ionization potential (I) and a high electron affinity (A) value that are most closely linked to HOMO and LUMO energy.

Target compounds have high χ, in order to 3g > 3e > 3h > 3f > 3a > 3c > 3d > 3b and high ω, in order to 3e > 3g > 3h > 3f > 3a > 3c > 3d > 3b. As seen, compound 3g (0.1439 eV) has a higher electronegativity and 3e (0.1213 eV) has a good electrophilic character than the others. According to the values of chemical hardness-softness (η, S), the compound having the high S and low η value is 3e has been determined.

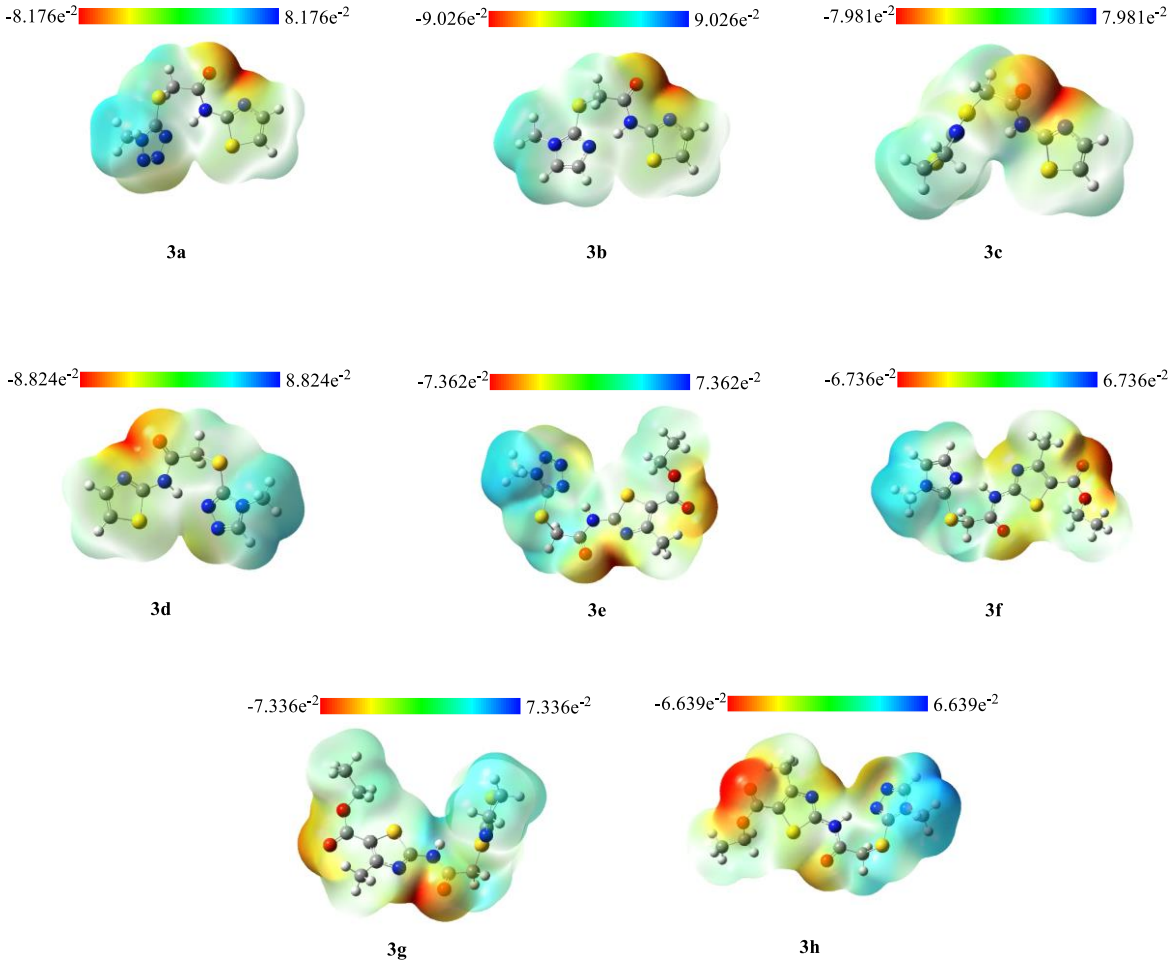


Figure 4: Molecular electrostatic potential (MEP) surfaces presentation of the compounds **3a-3h**

For the 3a–3h compounds, the molecular electrostatic potential (MEP) is shown in Figure 4. According to results, the density functional theory (DFT) calculations on 2-mercapto-*N*-(thiazol-2-yl)acetamide derivatives 3a-3h unveil crucial insights into their stability. The established order, with 3g demonstrating the highest stability, provides a foundation for understanding molecular behavior. This information is pivotal in predicting and rationalizing biological activities of these compounds. Stability is a key determinant in the overall efficacy and safety of drug candidates. The calculated values not only guide the selection of the most stable compounds but also offer valuable cues about potential reactivity. Consequently, DFT becomes an indispensable tool in drug development, aiding in the identification and optimization of compounds with favorable stability profiles that can contribute to enhanced biological activities and therapeutic outcomes.

Biologic activity results

In our study, we first investigated physicochemical properties, then performed a cytotoxic screening on three cancer cell lines and one healthy cell line to be sure that they had potential for further studies. Afterwards, we have achieved very important results.

The cytotoxic properties of the compounds 3a–3h were tested on human-derived A549 non-small cell lung cancer, Caco-2 colorectal cancer, SHSY-5Y neuroblastoma tumor cell lines, and NIH/3T3 mouse fibroblast cell line by using conventional MTT method. IC_{50} values were calculated, and the results were shown in Table 4. Doxorubicin was used as reference drug.

According to results, SHSY-5Y cancer cells were more sensitive than other cancer cells. Mostly, compound 3e has more potential than its analogs in the manner of cancer drug research. A few compounds determined as more active than standard drug, doxorubicin. These are compound 3e (IC_{50}: 46.40±0.43 μg.ml^{-1} and 10.43±0.31 μg.ml^{-1}) against A549 and SHSY-5Y cells, 3c (49.30±0.41 μg.ml^{-1}), 3f (11.87±0.33 μg.ml^{-1})) and 3g (10.74±0.29 μg.ml^{-1}) against SHSY-5Y cells. Overall, compounds did not show cytotoxicity against healthy cells at their IC_{50} values on cancer cells.

Table 4. IC_{50} (μg.ml^{-1}) values of the compounds (3a–3h) against A549, Caco-2, SHSY-5Y tumor and NIH/3T3 fibroblast cell lines

Compounds	A549	Caco-2	SHSY-5Y	NIH/3T3
3a	281.0±0.72	66.60±1.75	63.25±0.39	214.40±0.58
3b	388.80±0.64	284.10±0.76	363.3±0.49	436.30±0.68
3c	89.80±0.91	107.50±0.81	49.30±0.41	>500±0.47
3d	344.0±0.53	328.0±0.64	374.30±0.52	278.50±0.44
3e	46.40±0.43	233.0±0.82	10.43±0.31	291.20±0.64
3f	234.90±0.38	52.10±0.54	11.87±0.33	263.80±0.72
3g	250.20±0.52	205.0±0.77	10.74±0.29	>500±0.37
3h	308.30±0.60	243.0±0.69	71.00±0.48	179.80±0.68
Doxorubicin	86.25±0.25	42.10±0.38	60.20±0.42	202.75±0.29

DISCUSSION

The designed molecules (3a-3h) were synthesized purely with a high yield. The synthesis route is very useful and also cheap; therefore, it can be applied to reproduce the active compounds in a large scale. The drug-likeness of the synthesized compounds is high according to the results of the ADME calculations. With the exception of three compounds (3e, 3g and 3h), gastrointestinal absorption was predicted to be high for probable oral use of the compounds. The activity results indicated that compound 3d is more toxic to healthy cells than three cancer cells. Also, compound 3h is more toxic against healthy cells than A549 lung carcinoma and Caco-2 colorectal adenocarcinoma cells. These findings indicated that the *N*-methyl triazole ring system caused more cytotoxicity effect on NIH/3T3 healthy cells than other azole rings. Furthermore, the thiazoline ring (3c and 3g) had no impact on healthy cells. These two findings suggested together that mercapto azole moiety has a role in selectivity meanwhile aromaticity increases the cytotoxicity in healthy cells which is an unfavorable feature. Analyzing antitumoral activity on A549 cell line, compound 3e exhibited the highest cytotoxicity with selective profile which was better than doxorubicin. Compound 3c was also showed moderate cytotoxicity to same cell line. Meanwhile, in addition to 3d, and 3h, compound 3a is not safe to test for further tests because of high toxicity on healthy cells than A549 cells. Remain compounds (3b, 3f, and 3g) have a narrow IC_{50} range. Results of antitumoral activity on Caco-2 cell line showed that compounds 3a and 3f displayed the highest antiproliferative activity with selectivity. Except 3d and 3h, all compounds affected Caco-2 cells more than healthy cells. However, except for 3a and 3f, the remaining compounds have above 100 μg.ml^{-1} IC_{50} values, so only these two compounds were found valuable in this anti-Caco-2 activity. SHSY-5Y cell line was the most susceptible type against the tested compounds; 3c, 3e, 3f and 3g (IC_{50}: 10.43-49.3 μg.ml^{-1}) showed higher cytotoxicity than the standard drug (IC_{50}: 60.20 μg.ml^{-1}). Besides, compounds 3a and 3h showed remarkable antiproliferative activity. Only compound 3d had more cytotoxicity on SHSY-5Y cells than NIH/3T3 healthy cells; moreover, compounds 3e, 3f, and 3g have very low IC_{50} values (around 10 μg.ml^{-1}). These three compounds have an aryl ethyl carboxylate group, hence, this finding indicated that the ester group has a positive impact and is related to the anti-inflammatory and neuroprotective effects of this group as mentioned previously (Markovic et al. 2023; Osmaniye et al. 2023; Youdim 2013; Yucel et al. 2024). Meanwhile, there was a correlation between anticancer effects against Caco-2 and SHSY-5Y cell lines. It indicated that the anticancer activity on both cell lines of the compounds was affected similarly by the atoms or groups' replacement. On the other hand, it was not observed in this way for the A549 cell line, even most active compound was determined as 3e, compounds 3f and 3g were not effectiveness as much as 3e. So, we suggested that the mechanism of action for compounds against Caco-2 and SHSY-5Y are the same or similar, however, it's different against A549 cells. This difference is probably related to the insulin or ROS sensitivity of both cells as reported (Skora et al. 2022; Szychowski et al. 2019). To clarify this difference, new tests via experimental and computational approaches should be run. Unfortunately, because of the limitation of this study, we can only report potential anticancer agents and the structure-activity relationship in this study.

In summary, SHSY-5Y cells were more sensitive than other cell lines to the final compounds. Additionally, *N*-methyl triazole is not a favorable group since it increased cytotoxicity effect on healthy cells, contrary to the thiazoline ring system.

CONCLUSIONS

In this study, eight *N*-thiazole acetamide analogs were synthesized. Their structural analyses were identified by HRMS, ^{1}H-NMR and ^{13}C-NMR. Their physicochemical properties were calculated using *in silico* methods, and the anticancer activity was evaluated against A549 non-small lung epithelial carcinoma, Caco-2 colon carcinoma, and SHSY-5Y neuroblastoma cells while cytotoxicity effect was

tested against NIH/3T3 fibroblast cells. The results showed that compounds have good ADME profile and chemical stability of the targeted chemicals, compound 3g is the most stable. The predicted physicochemical properties of targeted compounds (3a-3h) indicate compliance with Lipinski's rule of five, suggesting good oral drug potential. Most compounds show high gastrointestinal absorption, with variations in molecular weight, hydrogen bonding, TPSA, and log P values, contributing to their pharmacokinetic profiles. Because it represents the energy balance inside a molecule, stability is important in chemical systems. Final compounds also have significant impact on SHSY-5Y cells with higher selectivity than other cells. Combination of ester group on thiazole and thiazoline (compound 3g) were found significantly effective than doxorubicin and highly selective on SHSY-5Y cells than healthy cells. Besides that, combination of thiazole-triazole (3d and 3h) decreased antiproliferative activity on three cancer cells while increased cytotoxicity on healthy cell. To understand the strength of activity against three cell lines, A549, Caco-2, and SH-SY5Y cell lines, have an important role in clarifying the mechanism of action. Meanwhile, Density Functional Theory (DFT) calculations on targeted compounds (3a–3h) provide critical insights into their stability and reactivity. The total energy values suggest that compound 3g is the most stable, while compound 3e, with the smallest energy gap, is the most reactive. Dipole moment analysis reveals varying polarity, with 3f being the most polarized and 3c the least. Additionally, compound 3e exhibits high chemical softness and low hardness, indicating significant reactivity. The calculated molecular electrostatic potentials further support these findings. These results highlight the utility of DFT in predicting and rationalizing the stability and reactivity of compounds, which are essential for drug development. By identifying the most stable and reactive molecules, DFT aids in the optimization of drug candidates, contributing to improved biological activities and therapeutic outcomes. In particular, insulin-dependent and ROS-related pathways were marked as major possible targets for these compounds. For further studies, these mechanisms will be investigated firstly by computer-aided approaches (CAAs), then experimental studies will be run according to the results from CAA. This study suggests that future perspectives in studies regarding the treatments of neuroblastoma and its related diseases of *ethyl 2-acetamido-4-methylthiazole-5-carboxylate* and thiazoline combination is encouraging.

Conflict of interest: The authors have no conflicts of interest to report.

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RESEARCH ARTICLE

In Vitro Antibacterial Effect of Hesperidin Microemulsion to *Staphylococcus aureus* and *Listeria monocytogenes*

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ABSTRACT

Foodborne bacterial infections and intoxications constitute a large proportion of bacterial diseases worldwide. In recent years, there has been an increasing resistance to chemotherapeutics used in the treatment of these diseases. Studies have shown that some compounds extracted from natural products and plants have alternative potential to antibiotics. In this study, it was aimed to reveal the antimicrobial potential of hesperidin microemulsion on *Listeria monocytogenes* and *Staphylococcus aureus*. In the study, the broth microdilution method was used to demonstrate *in vitro* antimicrobial activity. In the microdilution test, the MIC values of hesperidin for *S. aureus* and *L. monocytogenes* were as 128 µg/mL. In comparison, the MIC values of ampicillin for *S. aureus* and *L. monocytogenes* were as 0.5 µg/mL. Hesperidin is a compound with biologically valuable properties. Its antimicrobial effect may open a promising field as an alternative to antibiotics. However, detailed studies on hesperidin and similar potential active substances are needed to fully understand its therapeutic potential and to define its mechanisms of action.

Keywords: Antibacterial, Hesperidin, *Listeria monocytogenes,* MIC, *Staphylococcus aureus*

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Hesperidin Mikroemülsiyonunun *Staphylococcus aureus* ve *Listeria monocytogenes*'e Karşı *In Vitro* Antibakteriyel Etkisi

Gıda kaynaklı bakteriyel enfeksiyonlar ve zehirlenmeler dünya genelinde bakteriyel hastalıkların büyük bir bölümünü oluşturmaktadır. Son yıllarda bu hastalıkların tedavisinde kullanılan kemoterapötiklere karşı artan bir direnç söz konusudur. Yapılan çalışmalar, doğal ürünlerden ve bitkilerden elde edilen bazı bileşiklerin antibiyotiklere alternatif potansiyele sahip olduğunu göstermiştir. Bu çalışmada hesperidin mikroemülsiyonunun *Listeria monocytogenes* ve *Staphylococcus aureus* üzerindeki antimikrobiyal potansiyelinin ortaya konulması amaçlanmıştır. Çalışmada, *in vitro* antimikrobiyal aktiviteyi göstermek için broth mikrodilüsyon yöntemi kullanılmıştır. Mikrodilüsyon testinde hesperidinin *S. aureus* ve *L. monocytogenes* için MİK değerleri 128 µg/mL olarak bulunmuştur. Buna karşılık, ampisilinin *S. aureus* ve *L. monocytogenes* için MİK değerleri 0.5 µg/mL olarak bulunmuştur. Hesperidin biyolojik olarak değerli özelliklere sahip bir bileşiktir. Antimikrobiyal etkisi antibiyotiklere alternatif olarak umut verici bir alan açabilir. Bununla birlikte, terapötik potansiyelini tam olarak anlamak ve etki mekanizmalarını tanımlamak için hesperidin ve benzeri potansiyel aktif maddeler üzerinde ayrıntılı çalışmalara ihtiyaç vardır.

Anahtar Sözcükler: Antibakteriyel, Hesperidin, *Listeria monocytogenes*, MİK, *Staphylococcus aureus*

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INTRODUCTION

Infectious diseases are generally caused by waterborne and foodborne microbial agents. Among these, pathogenic bacteria such as *Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes, Campylobacter* spp. and *Escherichia coli* O157:H7 are the leading causes of foodborne gastroenteritis (Bailey et al. 2003; Hizlisoy et al. 2020). *S. aureus* is a pathogen that is often blamed for a variety of serious diseases such as cellulitis, endocarditis, and bacteremia from skin infections (Pal et al. 2023). *S. aureus* is commonly found on the skin and mucous membranes of farm animals. The causative bacterial agent causes poisoning from food prepared from the products of animals produced as food worldwide (Narayan et al. 2023). *L. monocytogenes* is also considered one of the most important zoonotic agents as it causes disease in both animals and humans. *L. monocytogenes* is found in nature, food, humans, animals, and plants (Lourenco et al. 2022). *L. monocytogenes* infections in humans are primarily caused by the consumption of contaminated food, leading to listeriosis, which is characterized by life-threatening meningitis, septicemia, and premature stillbirths (Schaefer et al. 2022). Cross-contamination has been recognized as the primary cause of contact of *L. monocytogenes* with food (Dos Santos et al. 2021). Macrolide, quinolone, tetracycline, gentamicin and beta-lactam group antibiotics are generally used in the treatment of foodborne bacterial infections (Ge et al. 2022). Resistance to these antibiotics is a serious global public health problem. In recent years, there has been an increasing development of resistance to many of the currently used antibiotics (Majumder et al. 2020). Alternatively, it is important to use compounds found in nature or obtained by various methods such as extraction as effective antibacterial agents (Guglielmi et al. 2020). Here, not only antimicrobial resistance, but also the easy availability of natural substances, their cheapness, the lack of technological infrastructure in undeveloped and developing countries, and negative thoughts about the side effects of synthetic substances are effective (Baytop 1999). In terms of natural antimicrobial compounds, plants occupy an important place (Ju et al. 2022). Alkaloids, terpenes and phenolic compounds, also called secondary metabolites, are substances found in plants that are known to have antimicrobial properties (Duraipandiyan et al. 2006). Among the phenolic compounds, flavonoids are widely found in plants. Flavonoids can also be found in various parts of plants, especially in fruit peels, leaves, flowers and seeds. In addition, there are subclasses such as flavonols, flavones, isoflavones, anthocyanins and catechins. Each subclass shows structural differences and may be specific to certain plant groups (Duraipandiyan et al. 2006).

Active metabolites in plants are obtained from plants by extraction methods. Extraction is the method used to separate a targeted substance from a mixture, concentrate an ingredient, or remove the desired substance from a material by physical and chemical methods (Jha and Sit 2022). Some common plant extraction methods include cold extraction, hot extraction, maceration, supercritical carbon dioxide extraction, ultrasonic extraction, and sublimation. Which extraction method to use may vary depending on the targeted active ingredient, plant material, and other factors. Furthermore, the solvent used and extraction times are also important factors influencing the results (Mathews et al. 2024).

Hesperidin (C16H14O6 3',5,7-trihydroxy-4'-methoxy flavanone), also a herbal extract, is a type of flavonoid and especially found in citrus peels and fruit juice. It is usually found in high amounts in citrus fruits such as lemons, oranges, tangerines, and grapefruits (Garg et al. 2001). Hesperidin has antioxidant properties with a high amount of flavonoids, and thanks to these properties, it can protect cells against the harmful effects of free radicals (de Souza et al. 2022). Besides, some studies also report that hesperidin may support cardiovascular health. In particular, hesperidin may increase blood vasodilation, which in turn may regulate blood pressure (Valls et al. 2021). Hesperidin has also the potential to regulate cholesterol levels (Altunayar-Unsalan et al 2022). In some studies, it has been shown that hesperidin can inhibit the development of cancer cells (Tan et al. 2020; Hermawan et al. 2021). However, research in this area is incomplete, and more studies are needed to determine whether they have similar effects in humans (Önder et al. 2023). Moreover, some studies have suggested that hesperidin has the potential to regulate blood sugar levels. (Shams-Rad et al. 2020).

In our study, it was aimed to investigate the *in vitro* antibacterial activities of hesperidin active ingredient on *S. aureus* and *L. monocytogenes*, which are important food pathogens.

MATERIALS and METHODS

Materials

The active ingredient hesperidin (Sigma-Aldrich Catalogue no.520-26-3), which was studied for the demonstration of antibacterial activity *in vitro*, was commercially available. Stock solutions of hesperidin were prepared in 100 mg/mL with DMSO (Dimethyl Sulfate Oxide) dilutions. The antibacterial properties of the active ingredient were compared with ampicillin (A9518, Merck, Germany) (Karayıldırım 2017). Again, *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 standard strains were used to determine antibacterial activity.

Bacterial Inoculum

To determine the antibacterial activity of hesperidin, *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 were used. The isolates were inoculated into blood agar with 7% sheep blood added (Merck, Germany Catalog number: 103879) and left to

incubate at 37°C for 18-24 hours. The reference strains were obtained from the culture collection of Erciyes University Faculty of Veterinary Medicine, Food Hygiene and Technology Laboratories.

Minimum Inhibitory Concentration (MIC) Test

A broth microdilution test was performed to demonstrate the antimicrobial efficacy of hesperidin against test bacteria (NCCLS 2007). In this method, briefly; at the end of 24 hours of incubation in an aerobic environment at 37°C, the fresh culture was inoculated into Mueller-Hinton broth (Merck, Germany) and the bacterial suspension was adjusted according to the 0.5 McFarland (~$1.5x10^8$ cfu/mL) standard was added to 100 µL equal volumes of 96-well microtiter plates. Prepared hesperidin stock solutions were added to 96-well microtiter plates and 1/2 serial dilutions were made. The dose was adjusted so that the hesperidin concentration in the wells ranged from 256 to 0.5 µg/mL. In the last well, 100 µL of liquid medium and 100 µL of bacterial suspension were used as negative control. All plates were covered with a sterile plate lid and incubated at 37 °C for 24 hours. The existing suspensions in the wells were planted in Mueller Hinton agar and left for incubation.

Wells containing dilutions of the ampicillin (A9518, Merck, Germany) as a positive control were adjusted to range from 4 to 0.36 µg/mL and were incubated in the same way. Additionally, controls were established for sterility, microorganism viability, and inhibitory activity of DMSO.

Statistical Analysis

The results of MIC values of Hesperidin against to *S. aureus* and *L. mononocytogenes* were expressed in mean+standard deviation using Microsoft Office Excel 2019 (Microsoft Corporation, Redmond, WA, USA). Significant differences between the averages were determined by the T-test (SPSS for Windows 11)

RESULTS

According to the results of the microdilution test, the MIC values of *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 bacteria were determined as 128 µg/mL and 128 µg/mL for hesperidin. In addition to these results, the MIC values of *S. aureus* and *L. monocytogenes* bacteria were determined as 0.5 µg/mL for ampicillin, respectively (Table 1). As a result of the T test, it was seen that there was no significant difference in terms of the MIC values of the bacteria.

Table 1. MIC values of hesperidin and ampicillin against *S. aureus* and *L. monocytogenes*

ATCC	Hesperidin	Ampicillin
S. aureus	128 µg/mL	0.5 µg/mL
L. monocytogenes	128 µg/mL	0.5 µg/mL

DISCUSSION

In recent years, the use of natural products in the treatment of infectious diseases has been increasing (Newman and Cragg, 2020). Among the main reasons for this increase are the side effects of synthetic drugs and the antimicrobial resistance of microorganisms against these drugs (Malekzadeh et al. 2001, Nostro et al. 2005). Around the world, interest in research examining the antimicrobial properties of extracts from plants has increased in parallel (Chassagne et al. 2021). Many plants such as *Alpinia galanga, Anethum graveolens, Asperugo procumbens, Bixa orellana, Feoniculum vulgare, Phyllanthus emblica* and *Vitis vinifera* have been examined for their antimicrobial properties around the world and in our country (Ozen et al. 2020). Alkaloids, terpenes, and phenolic compounds in medicinal plants are compounds that show antimicrobial activity in plants (Duraipandiyan et al. 2006). Hesperidin, a flavanone compound, is of medical importance due to its antioxidant, anti-inflammatory and antibacterial properties. Pharmacological studies have shown that hesperidin is a potent antimicrobial, analgesic, and immunomodulatory agent (Tejada et al. 2018).

In the study conducted by Abuelsaad et al. (2013), the antimicrobial activity of hesperidin against *Aeromonos hydrophila* was tested by broth microdilution method and as a result of the examination, it was revealed that hesperidin was active against *A. hydrophila* at doses of 100, 50, 25 and 12.5 mg/mL. In the studies of Ghorab and Ibraheim (2018), the antibacterial activity of hesperidin on *Streptococcus mutans* was investigated by agar diffusion method and the presence of an inhibition zone against bacteria was revealed at the end of the test.

In the study conducted by Karayıldırım (2017) in Turkiye, hesperidin microemulsion was tested in terms of antibacterial activity against various Gram-

positive and negative bacteria. At the end of the study, it was stated that hesperidin showed strong activity on *E. coli* (8 µg/mL) and *E. faecalis* (16 µg/mL) at a concentration equal to standard antibiotic gentamicin. Again, in the study conducted by Çetinkaya et al. (2019), it was reported that the application of hesperidin to patients with acute otitis media for 14 days reduced the symptoms of the disease with an effect similar to antibiotics.

In our study, according to the results of the MIC test performed to reveal the antimicrobial activity of hesperidin, the MIC values of *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 bacteria were found to be 128 µg/mL for hesperidin. In addition, MIC values of *S. aureus* and *L. monocytogenes* bacteria were determined as 0.5 µg/mL for ampicillin. Accordingly, although it is understood that the antibiotic ampicillin is effective at a lower titer, it is important that hesperidin, which is a natural product, also has antimicrobial activity.

The efficacy of its active compounds in plants varies depending on the collection, drying, and storage processes (Baytop 1999, Zaidan et al. 2005). There is a period when the pharmaceutical active compound of each plant is at the highest level (Baytop 1999). Therefore, plants should be harvested during periods when the active ingredient they contain is highest (Atata et al. 2003). The herbal active ingredient used in the study is a commercial microemulsion and is not affected by external factors to which other herbal metabolites are exposed if stored under appropriate conditions.

CONCLUSION

In conclusion, hesperidin is a biologically valuable compound. Its antimicrobial activity is a promising situation for alternative drug research to antibiotics. However, in order to discover its full therapeutic potential and to define its mechanisms of action in the later stages, detailed studies are needed on active ingredients with high potential as an alternative to hesperidine and similar antibiotics.

Conflict of interest: The author has no conflicts of interest to report.

Authors' Contributions: HH contributed to the project idea, design, execution, data acquisition and analysis of the study.

Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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RESEARCH ARTICLE

Nutritional Properties of Different Types of Cherry Laurel Fruits and Seeds

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ABSTRACT

Cherry laurel (Prunus laurocerasus) is an evergreen tree, approximately 5-6 meters tall, with small white flowers, belonging to the Rosaceae family, which is frequently found in the Black Sea region in Türkiye. It has fruits with conical hard seeds resembling cherries, in clusters like grapes, and in red to purplish black colors when ripe for eating. In Türkiye, the fruits, leaves and seeds of cherry laurel are used as a folk medicine for many ailments. This study aimed to determine some physicochemical properties (weight, width, length, seed weight, moisture, ash, pH, protein, fat, glucose, fructose and dietary fiber) of different types of cherry laurel fruits and seeds grown in Türkiye. Weight, width, length, moisture, ash, pH, protein, glucose, fructose and dietary fiber of different types (61K04 (Trabzon), 14K02 (Bolu), 28K13 (Giresun), 08K02 (Artvin)) cherry laurel fruits contents were determined as 3.0108-5.753 g; 15.107-20.813 mm; 17.179-19.884 mm; 65.267-75.712%; 0.567-1.005%; 3.820-4.260; 0.735-0.942%; 9.077-12.155 g.100g^{-1}; 3.605-7.176 g.100g^{-1} and 16.160-18.075% respectively. When the seeds of cherry laurel fruits are examined, the shell weight is 0.123-0,174 g, inner seed weight is 0.065-0.110 g, moisture is 4.020-6.301%, ash is 2.722-4.173%, pH is 5.415-5.730, protein is 17.693-26.051%, fat 25.773-33.800%, glucose 4.841-10.958 g.100 $^{-1}$, fructose 0.584-2.581 g.100g^{-1} and dietary fiber was found between 16.505-26.560%. In line with these results; It has been determined that cherry laurel fruits and seeds have different basic nutritional properties depending on their type.

Keywords: Cherry Laurel, Fruit, Physico chemical properties, Prunus laurocerasus, Seed

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Farklı Tip Karayemiş Meyve ve Çekirdeklerinin Besinsel Özellikleri

ÖZ

Karayemiş (Prunus laurocerasus) Türkiye'de Karadeniz bölgesinde sıklıkla rastlanan Rosaceae familyasına ait yapraklarını dökmeyen yaklaşık 5-6 metre boylarında küçük beyaz çiçekleri olan bir ağaçtır. Kiraza benzeyen konik sert çekirdekli, üzüm gibi salkım halinde, yeme olgunluğuna geldiğinde kırmızı ile morumsu siyah renklerinde meyveleri vardır. Türkiye'de karayemişin meyveleri, yaprakları, çekirdekleri bir halk ilacı gibi pek çok rahatsızlıklarda kullanılmaktadır. Bu çalışma Türkiye'de yetişen farklı tipteki karayemiş meyve ve çekirdeklerinin bazı fizikokimyasal özelliklerinin (ağırlık, en, boy, çekirdek ağırlığı, nem, kül, pH, protein, yağ, glukoz, fruktoz ve diyet lifi) belirlemesi amaçlanmıştır. Farklı tip (61K04 (Trabzon), 14K02 (Bolu), 28K13 (Giresun), 08K02 (Artvin)) karayemiş meyvelerinin ağırlık, en, boy, nem, kül, pH, protein, glukoz, fruktoz ve diyet lifi içerikleri sırasıyla 3,0108-5,753 gr; 15,107-20,813 mm; 17,179-19,884 mm; %65,267-75,712; %0,567-1,005; %3,820-4,260; %0,735-0,942; 9,077-12,155 g.100g^{-1}; 3,605-7,176 g.100g^{-1} ve %16,160-18,075 olarak tespit edilmiştir. Karayemiş meyvelerinin çekirdekleri incelendiğinde kabuklu ağırlık 0,123-0,174 gr, çekirdek iç ağırlığı 0,065-0,110 gr, nem %4,020-6,301, kül %2,722-4,173, pH 5,415-5,730, protein %17,693-26,051, yağ %25,773-33,800, glukoz 4,841-10,958 g.100g^{-1}, fruktoz 0,584-2,581 g.100g^{-1}, ve diyet lifi %16,505-26,560 arasında tespit edilmiştir. Bu sonuçlar doğrultusunda; karayemiş meyve ve çekirdeklerinin tiplerine göre farklı temel besinsel özelliklere sahip olduğu belirlenmiştir.

Anahtar kelimeler: Çekirdek, Fizikokimyasal Özellikler, Karayemiş, Meyve, Prunus Laurocerasus

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GİRİŞ

Bitki çeşitliliği bakımından önemli bir ülke olan Türkiye'de çok sayıda farklı meyve türleri bilinmekte ve yetiştirilmektedir (İslam ve ark. 2010). Ülkemiz pek çok meyve türü için bir ana vatan, meyvecilik kültürü açısından da önemli bir merkez konumundadır. Karadeniz Bölgesi çok sayıda değişik bitki ve meyvelerin olduğu çeşitlilik ve zengin doğal kaynaklar açısından önemli bir bölgedir (İslam ve Deligöz 2012). Karayemiş de (*Laurocerasus officinalis* Roem.) Karadeniz Bölgesi kıyılarında yaygın olarak bulunan bir meyvedir ve yerel olarak "Taflan" şeklinde de adlandırılır (Alasalvar ve ark. 2005; Liyana-Pathirana ve ark. 2006). Doğu Karadeniz bölgesinde sıklıkla rastlanan *P. Laurocerasus*, Rosaceae familyası ve Prunoideae subfamilyasına aittir (Kolaylı ve ark. 2003).

Karayemiş Türkiye'de Doğu Karadeniz bölgesinde, doğu Marmara'da, Bazı Akdeniz ülkelerinde, Kuzey İrlanda, Batı Avrupa, Güney ve Batı Kafkaslar, İran, Balkanların bir kısmı ve bazı Akdeniz ülkelerinde yetişmektedir. Doğu Karadeniz bölgesinde yaygın olarak tüketilmektedir (Kolaylı ve ark. 2003).

Karayemiş ağacı yapraklarını dökmeyen bir ağaçtır ve bu sebeple çölleşmenin engellenmesinde fayda sağlarken, yaprakları da peyzaj mimarisinde kullanım alanı bulmaktadır (Kolaylı ve ark. 2003). Büyük, parlak ve koyu yeşil renkte yaprakları olan karayemiş yaklaşık 5-6 metre boylarında küçük beyaz çiçekleri olan bir ağaçtır. Mart ayında çiçeklenip Haziran-Temmuz aylarında hasat edilebilir. Kiraza benzeyen konik sert çekirdekli, üzüm gibi salkım halinde meyveleri vardır. Meyveler parlak, pürüzsüz görünümdedir. Yeme olgunluğuna geldiğinde kırmızı ile morumsu siyah renklerindedir. Meyveler olgunlaşmadan önce tatları buruktur, olgunlaşma ilerledikçe aromatik ve taze olarak tüketilebilir bir hale gelir (İslam, 2002). Meyvelerin fiziksel ve kimyasal özellikleri türlere göre farklılık göstermektedir (Sulusoglu ve ark. 2015).

Genel olarak meyveler taze olarak tüketilir fakat bunun yanında reçel, turşu ve kek yapımında da kullanılır. Meyvelerin kurutulup tüketilmesi de yaygın bir kullanım şeklidir (Sülüşoğlu ve Cavuşoğlu 2011; Sulusoglu ve ark. 2015). Ülkemizde karayemişin meyveleri, yaprakları, çekirdekleri bir halk ilacı gibi mide ve sindirim sistemi rahatsızlıklarında, bronşitte, cilt rahatsızlıklarında ve hemoroit hastalığında yaygın olarak kullanılmaktadır. Yaprakları ateş düşürücü ve analjezik olarak kullanılmaktadır (Yeşilada ve ark. 1999). Karayemiş öksürük kesici, idrar sökücü ve spazm çözücü olarak bronşit, böbrek taşı, sindirim sistemi hastalıkları, mide ülseri, hemoroit, egzama gibi rahatsızlıklarda kullanılmaktadır (Erdemoglu ve ark. 2003; Karahalil ve Sahin 2011; Ozturk ve ark. 2017). İnsan metabolizmasında meyvelerin bağışıklığı kuvvetlendirici etkisi olduğuna inanılır (Karahalil ve Şahin 2011). Alasalvar ve ark. (2005), karayemişin gıda ve gıda takviye formülasyonlarında kullanılabilecek iyi bir antioksidan kaynağı olduğunu belirtmişlerdir. Pek çok hastalığın önlenmesinde ve tedavisinde, yapısında bulunan antioksidan özellikli maddeler, flavonoidler, fenolik bileşikler ve yağ asitlerinin sayesinde etkili olabileceği belirtilmektedir (Alasalvar ve ark. 2005; Karataş ve Uçar 2018).

Orhan ve ark. (2015) tarafından karayemiş meyve, çekirdeksiz meyve ve çekirdeklerinin diyabet hastalığı üzerine etkileri ile ilgili ratlarla yapılan çalışmada karayemiş çekirdeğinden hazırlanan özütün, antidiyabetik etkisinin olduğu tespit edilmiştir. Doğu (2014), tarafından yapılan çalışmada ise *Prunus laurocerasus* ekstraktının tek olarak ve metforminle birlikte uygulanmasında antihiperlipidemik, antihiperglisemik, ve antioksidan etki gösterdiği, ve diyabetin meydana getirebileceği oksidatif hasarı önleyebilme özelliğinden dolayı diyabet tedavisinde destekleyici olarak kullanılabileceği belirtilmiştir.

Karayemiş meyvesinin 100 gramı içerisinde, 75 kcal enerji, 79,85 gr su, 14,72 gr karbonhidrat, 0,68 gr kül, 1,40 gr protein, 2,81 gr diyet lifi, 5,09 glukoz, 6,36 gr fruktoz, 0,55 mg demir, 2,3 mg C vitamini ve 2 RE (Retinol Eşdeğeri) A vitamini bulunduğu bildirilmiştir (TürKomp 2024). Karadeniz bölgesinde yetişen sekiz adet karayemiş genotipinin kuru maddede fenolik madde içeriği Halilova ve Ercisli (2010) tarafından 24,36 ve 75,27 mg/100 g arasında belirtilmiştir. Yedi farklı karayemiş genotipinde yapılan başka bir araştırmada ise antioksidan aktivesi DPPH testine göre 1,07-12,19 mmol TE g^{-1} aralığında tespit bildirilmiştir (İslam ve ark. 2020). Celep ve ark. (2012) tarafından yenilebilir üç farklı meyvenin antioksidan potansiyellerinin incelendiği bir çalışmada karayemiş meyvesinin toplam fenolik madde içeriği 23,64±0,84 mg GAE.gr^{-1} ekstrakt, toplam flavonoid içeriği 16,87±0,38 (mg QE.gr^{-1} ekstrakt) ve toplam proantosiyanidin içeriği ise 342±16,3 mg EGCG-E/ekstrakt olarak bildirilmiştir.

Karayemiş meyvesi (*Prunus laurocerasus*) yaprak ve meyve ekstraktlarının kompozisyonu ve antioksidan aktivitesi üzerine farklı ekstraksiyon tekniklerinin etkisinin araştırıldığı bir çalışmada total fenolik içeriği 36,2±0,6 - 46,3±1,4 mg gallik asit.g^{-1} kuru ekstrakt, toplam flavonoid içeriği 12,9±0,2 - 13,7±0,5 mg rutin.g^{-1}kuru ekstrakt olarak belirtilmiştir (Karabegovi´c ve ark. 2014).

Literatür taramaları doğrultusunda yapılan çalışmaların daha çok karayemiş meyve kısmıyla ilgili olduğu görülmektedir. Bu doğrultuda araştırmamızda; Türkiye'de yetişen farklı tipteki karayemiş meyve ve çekirdeklerinin bazı fiziksel, kimyasal ve besleyici özelliklerinin belirlenmesi amaçlanmıştır.

MATERYAL ve METOD

Materyal

Bu araştırma kapsamında Karadeniz Tarımsal Araştırmalar Enstitüsünden dört farklı tipte (61K04, 14K02, 28K13 ve 08K02) karayemiş meyveleri temin edilmiştir. Örneklerde kullanılan isimlendirme sistemi

Karadeniz Tarımsal Araştırmalar Enstitüsünde kayıtlı oldukları şekliyle kullanılmıştır. Çalışmamızda isimlendirme sistemine göre 61K04 Trabzon, 14K02 Bolu, 28K13 Giresun ve 08K02 Artvin ilinden toplanılıp çelikle köklendirilip Karadeniz Tarımsal Araştırma Enstitüsü bahçesinde yetiştirilen ağaçların meyveleri kullanılmıştır. Temin edilen yaş meyveler ve uygun şartlarda (gölgede) kurutulan meyve ve çekirdekleri ayrı ayrı analizlere tabi tutulmak üzere muhafaza edilmiştir. Çekirdekte yapılacak analizler için çekirdekler meyve etinden ayrılıp kabukları kırılarak çekirdek içleri çıkarılmıştır.

Metot

Farklı Tip Karayemiş Meyve ve Çekirdeklerinde Yapılan Analizler

Bu doğrultuda karayemiş çekirdek ve meyvelerin ağırlıkları 0,001 gr' a duyarlı hassas terazide tartılmış ve ortalaması alınarak belirlenmiştir. Meyve örneklerinin en (mm) ve boyları (mm), rastgele alınan örneklerin en geniş ve en uzun kısımları arasında olmak üzere 0,01 mm'ye duyarlı dijital kumpas ile ölçülmüştür. Diğer taraftan karayemiş meyve ve çekirdeklerinde nem analizi TS 1129-ISO 1026 (TSE, 1998), kül analizi TS ISO 763 (TSE, 2015), pH analizi TS 1728 ISO 1842 (TSE, 2001) ve protein analizi TS 1620 (TSE, 2016) standart metotlarına göre belirlenmiştir.

Yağ Miktarı Tayini

Karayemiş meyvelerinin yağ miktarı analizleri FAO Food Quality Control (1986) metoduna göre yapılmıştır. Karayemiş çekirdeklerinin yağ miktarı analizinde ise karayemiş çekirdekleri öğütme işlemine tabi tutulduktan sonra, soxhelet cihazı ile yağ oranlarının tespiti yapılmıştır. Bu amaç ile 10'ar gr öğütülmüş numuneler LAB 312 model yarı otomatik soxhelet cihazına koyularak her bir numune üzerine 50 mililitre hekzan eklenmiştir. Daha sonra sırasıyla 85 °C de 50 dakika çözücü hekzan ile yıkama, 80 °C sıcaklığında 40 dakika kaynatma ve 80 °C 20 dk çözücü geri kazanımı işlemi uygulanarak, elde edilen karayemiş çekirdek yağları desikatörde oda sıcaklığına kadar soğutma işlemi gerçekleştirilerek, tartma işlemi yapılmış ve sonuçlar kuru madde üzerinden hesaplanarak, karayemiş çekirdeklerinin % yağ oranları belirlenmiştir (TSE, 2000).

Şeker Analizleri

Bu kapsamda karayemiş meyve ve çekirdeklerinde glukoz, fruktoz ve sakkaroz bileşenlerinin analizi gerçekleştirilmiştir. Bu analizler HPLC sistemi kullanılarak AOAC 980.13'e göre yapılmıştır. Örnekler analize alınmadan önce karıştırılarak veya öğütülerek homojen hale getirilmiştir. 5 gram numune, 50 ml'lik balonjojeye tartılıp 25 ml su ile çözülüp asetonitril ile hacme tamamlanmıştır. 0.45µm'lik filtreden geçirilip HPLC vialine alınarak analiz edilmiştir. Cihazın çalışma şartları: Enjeksiyon hacmi 1.5µl, akış hızı: 0.2 ml/dak.'dır. HPLC sisteminde NH_2P-50 2D, 150X2 mm özelliklerinde kolon kullanılmıştır (AOAC, 1980).

Diyet Lifi Analizi

Kurutulmuş karayemiş meyve ve çekirdeklerindeki diyet lifi miktarı, %10 dan fazla yağ içeren numunelerin yağları alındıktan sonra, enzimlerle sindirilebilir kısımların uzaklaştırılması ve çözünür diyet lifinin alkol ile çöktürülmesi neticesinde kalan diyet lifinin kurutulup miktarının belirlenerek kül ve protein içeriğine göre sonucun belirlenmesi prensibine göre olan AOAC 985.29 metoduna göre belirlenmiştir (AOAC, 1985).

İstatiksel Analiz

Minitab 21.0 programı ile verilerin istatistiki değerlendirmeleri yapılmıştır. Bu kapsamda, verilerin ortalama standart sapma değerleri belirlenmiş ve Tek Yönlü Anova testi ile ikiden çok bağımsız grup arasında niceliksel sürekli verilerin karşılaştırılması yapılmıştır (Püskülcü ve İkiz 1998).

BULGULAR

Bu çalışma kapsamında Türkiye'de yetişen farklı tip karayemiş meyve ve çekirdeklerinin fiziksel ve belirli temel besinsel analiz sonuçları (ağırlık, en, boy, nem, kül, pH, protein, yağ, şeker ve diyet lifi) sırasıyla aşağıda belirtilmiştir.

Tablo 1'de görüldüğü üzere, kodları belirtilen farklı tipteki karayemiş meyvelerinin sırasıyla ağırlık, en ve boy değerleri; 61K04 (Trabzon) (4,252 gr, 18,466 mm ve 19,073 mm), 14K02 (Bolu) (3,011 gr, 15,107 mm, 17,543 mm), 28K13 (Giresun) (3,376 gr, 16,234 mm, 17,179 mm) ve 08K02 (Artvin) (5,753 gr, 20,813 mm, 19,884 mm) olarak belirlenmiştir. Karayemiş meyveleri ağırlık (g) ve en (mm) değerleri açısından $p < 0,05$ düzeyinde istatistiki olarak incelendiğinde; 14K02 ve 28K13 karayemiş meyvelerinin birbirleri ile benzerlik gösterirken, 61K04 ve 08K02 karayemiş meyvelerinin farklılık gösterdiği belirlenmiştir. Boy (mm) açısından incelendiğinde ise; 61K04 ve 08K02 karayemiş meyvelerinin birbirleri ile benzerlik gösterdiği, 14K02 ve 28K13 karayemiş meyvelerinin de birbirleri ile benzerlik gösterdiği belirlenmiştir.

Tablo 1'de görüldüğü üzere en yüksek nem değerinin 08K02 (Artvin), en düşük nem değerinin ise 14K02 (Trabzon) kodlu karayemiş meyvesinde ölçülmüştür. Kül miktarı ve pH değeri açısından en yüksek değerin 14K02 (Bolu) kodlu karayemiş meyvesinde olduğu, en düşük kül miktarının 08K02 (Artvin) kod numaralı, en düşük pH değerinin ise 28K13 (Giresun) kod numaralı üründe olduğu tespit edilmiştir.

Karayemiş meyveleri nem değerleri ve pH değerleri açısından $p<0,05$ düzeyinde istatistiki olarak incelendiğinde; 61K04 ve 14K02 karayemiş tipleri birbirleri ile benzerlik gösterirken, 28K13 ve 08K02 karayemiş tiplerinin farklılık gösterdiği belirlenmiştir. Karayemiş meyveleri kül içerikleri açısından ise

p<0,05 düzeyinde istatistiki olarak 61K04, 28K13 ve numaralı karayemiş meyveleri birbirleri ile benzerlik gösterirken 14K02 karayemiş meyvesinin farklılık gösterdiği belirlenmiştir. Tablo 1' de görüldüğü üzere en yüksek protein değeri 28K13 (Giresun) kod numaralı karayemiş meyvesinde tespit edilirken, en düşük protein değerinin ise 61K04 (Trabzon) kod numaralı üründe olduğu belirlenmiştir. Karayemiş meyveleri protein değerleri açısından p<0,05 düzeyinde istatistiki olarak incelendiğinde; 14K02 ve 08K02 karayemiş meyvelerinin birbirleri ile benzerlik gösterirken 61K04 ve 28K13 karayemiş meyvesinin protein içerikleri yönünden farklılık gösterdiği belirlenmiştir. 14K02 karayemiş meyvesinin protein içeriği bakımından, 28K13 ve 61K04 karayemiş meyveleri ile kısmen benzerlik gösterdiği; 08K02 karayemiş meyvesinin protein içeriği bakımından ise 28K13 ve 61K04 karayemiş meyveleri ile kısmen benzerlik gösterdiği belirlenmiştir. Meyve örneklerinin tamamında yağ tespit edilmemiştir.

Tablo 2' de görüldüğü üzere çalışmamızda farklı tip karayemiş meyvelerinde en fazla glukoz 61K04 (Trabzon) kod numaralı karayemiş meyvesinde tespit edilirken, en düşük glukoz 08K02 (Artvin) kod numaralı meyvede tespit edilmiştir. Fruktoz içerikleri incelendiğinde ise yine en düşük fruktoz miktarı 08K02 (Artvin) kod numaralı meyvede tespit edilirken, en yüksek fruktoz 14K02 (Bolu) kod numaralı meyvelerde tespit edilmiştir. Hiçbir karayemiş meyve örneğimizde sakkaroz tespit edilememiştir. Karayemiş meyveleri fruktoz değerleri açısından p<0,05 düzeyinde istatistiki olarak incelendiğinde; 61K04 ve 14K02 karayemiş meyvelerinin fruktoz içerikleri yönünden birbirleri ile benzerlik gösterirken, 28K13 ve 08K02 karayemiş meyvesinin fruktoz içerikleri yönünden farklılık gösterdiği belirlenmiştir. Karayemiş meyveleri glukoz değerleri açısından incelendiğinde ise ; 61K04, 14K02 ve 28K13 karayemiş meyvelerinin glukoz içerikleri yönünden birbirleri ile benzerlik gösterirken; 08K02 karayemiş meyvesinin glukoz içerikleri yönünden farklılık gösterdiği belirlenmiştir.

Tablo 2' de görüldüğü üzere çalışmamızda 61K04 (Trabzon) ,14K02 (Bolu), 28K13 (Giresun) ve 08K02 (Artvin) kod numaralı karayemiş meyvelerinde sırasıyla %18,070±0,014, %18,075±0,049 , %16,160 ±0,028, %17,925±0,078 oranlarında diyet lifi tespit edilmiştir. Karayemiş meyveleri diyet lifi değerleri açısından istatistiki olarak incelendiğinde; 61K04 (Trabzon), 14K02 (Bolu) ve 08K02 (Artvin) karayemiş meyvelerinin diyet lifi içerikleri yönünden birbirleri ile benzerlik gösterirken, 28K13 (Giresun) karayemiş meyvesinin diyet lifi içeriği yönünden farklılık gösterdiği belirlenmiştir.

Tablo 1. Farklı tip karayemiş meyvelerine ait temel kompozisyonel analizler
Table 1. Basic compositional analyzes of different types of cherry laurel fruits

Analizler	61K04 (Trabzon)	14K02 (Bolu)	28K13 (Giresun)	08K02 (Artvin)
Ağırlık (gr)	4,252±0,784^{b}	3,011±0,2526^{c}	3,376±0,3001^{c}	5,753±0,676^{a}
En (mm)	18,466±2,436^{b}	15,107±0,643^{c}	16,234±0,746^{c}	20,813±1,442^{a}
Boy (mm)	19,073±1,565^{a}	17,543±0,730^{b}	17,179±0,680^{b}	19,884±1,057^{a}
Rutubet (%)	65,399±0,401^{c}	65,267±0,052^{c}	70,072±0,160^{b}	75,712±0,110^{a}
Kül (%)	0,617±0,080^{b}	1,005±0,080^{a}	0,622±0,074^{b}	0,567±0,067^{b}
pH	4,250±0,000^{a}	4,260±0,000^{a}	3,820±0,000^{c}	3,955±0,007^{b}
Protein (%)	0,735±0,025^{b}	0,802±0,081^{ab}	0,942±0,039^{a}	0,872±0,002^{ab}
Yağ (%)	T.E.	T.E.	T.E.	T.E.

$^{a-c}$ Farklı harfle işaretlenmiş ortalamalar istatistiki olarak (p<0,05) biri birinden farklıdır. ±: Standart sapma. T.E.: Tespit edilemedi.

Tablo 2. Farklı tip karayemiş meyveleri glukoz, fruktoz, sakkaroz ve diyet lifi içeriği
Table 2. Glucose, fructose, sucrose and dietary fiber content of different types of cherry laurel

Karayemiş Meyve	Glukoz (gr/100gr)	Fruktoz (gr/100gr)	Sakkaroz (gr/100gr)	Diyet Lifi (%)
61K04 (Trabzon)	12,155±0,064^{a}	6,620±0,052^{a}	T.E.	18,070±0,014^{a}
14K02 (Bolu)	11,467±0,714^{a}	7,176±0,307^{a}	T.E.	18,075±0,049^{a}
28K13 (Giresun)	11,839±0,088^{a}	4,541±0,175^{b}	T.E.	16,160±0,028^{b}
08K02 (Artvin)	9,077±0,109^{b}	3,605±0,061^{c}	T.E.	17,925±0,078^{a}

$^{a-c}$ Farklı harfle işaretlenmiş ortalamalar istatistiki olarak (p <0,05) biri birinden farklıdır. ±: Standart sapma. T.E.: Tespit edilemedi.

Tablo 3' de farklı tip karayemiş çekirdeklerine ait fizikokimyasal özellikler sunulmuştur. Bu kapsamda meyve çekirdeklerinin kabuklu ağırlık, iç ağırlık, rutubet, kül, pH, protein ve yağ içerikleri belirlenmiştir. Karayemiş meyvelerinin çekirdekleri, kabuklu ağırlık ve iç çekirdek ağırlığı yönünden incelendiğinde en yüksek değerlerin 08K02 (Artvin) kod numaralı karayemiş meyvesinin olduğu , en düşük değerlere ise kabuklu ağırlık açısından 14K02 (Bolu) kod numaralı karayemiş meyvelerinin, çekirdek iç ağırlığı açısından ise 61K04 (Trabzon) kod numaralı karayemiş çekirdeklerine ait olduğu belirlenmiştir.

Karayemiş çekirdekleri kabuklu ağırlık(gr) değerleri açısından $p<0,05$ düzeyinde istatistiki olarak incelendiğinde; 61K04, 14K02, 28K13 ve 08K02 birbirleri ile benzerlik gösterdiği belirlenmiştir. İç ağırlık (gr) değerleri açısından incelendiğinde; 61K04, 14K02 ve 28K13 karayemiş çekirdeklerinin birbirleri ile benzerlik gösterdiği belirlenmiştir. Ayrıca 08K02 karayemiş çekirdeklerinin de iç ağırlık ölçümleri yönünden birbirleri ile farklılık gösterdiği tespit edilmiştir.

Çalışmamızda incelediğimiz karayemiş tiplerinin çekirdeklerine ait analiz sonuçlarına göre en yüksek nem miktarı 14K02, en düşük nem miktarı 28K13 (Giresun) kod numaralı karayemiş örneklerine ait olduğu tespit edilmiştir. Karayemiş çekirdekleri nem değerleri açısından $p<0,05$ düzeyinde istatistiki olarak incelendiğinde; 61K04 ve 14K02 karayemiş tipleri birbirleri ile benzerlik gösterirken, 28K13 ve 08K02 karayemiş tiplerinin farklılık gösterdiği belirlenmiştir.

Kül değeri açısından çalışmamızdaki karayemiş tiplerinden en yüksek değer 14K02 (Bolu), en düşük değer ise 28K13 (Giresun) kodlu örnekte tespit edilmiştir. İstatistiki olarak incelendiğinde; 28K13 (Giresun) ve 08K02 (Artvin) karayemiş çekirdeklerinin kül içerikleri yönünden birbirleri ile benzerlik gösterirken, 61K04 (Trabzon) ve 14K02 (Bolu) karayemiş çekirdeklerinin kül içerikleri yönünden farklılık gösterdiği belirlenmiştir.

Tablo 3'de görüldüğü üzere karayemiş çekirdeklerinde pH değerleri 28K13 (Giresun) (5,730) en yüksek olarak belirlenmişken 61K04 (Trabzon), 14K02 (Bolu) ve 08K02 (Artvin) tiplerinde sırasıyla pH değerleri 5,415, 5,630, 5,660 olarak tespit edilmiştir. Karayemiş çekirdekleri pH değerleri açısından $p<0,05$ düzeyinde istatistiki olarak incelendiğinde; 61K04, 14K02, 28K13 ve 08K02 karayemiş tiplerinin pH yönünden farklılık gösterdiği belirlenmiştir.

Farklı tipteki karayemiş çekirdeklerinin protein oranları incelendiğinde en yüksek protein değeri 08K02 (Artvin) kodlu karayemiş çekirdeğinde belirlenmiş iken, en düşük protein değeri 28K13 (Giresun) kodlu karayemiş çekirdeklerinde tespit edilmiştir. Karayemiş çekirdekleri protein değerleri açısından $p<0,05$ düzeyinde istatistiki olarak incelendiğinde; 61K04, 28K13 ve 08K02 karayemiş çekirdeklerinin protein içerikleri yönünden birbirleri ile farklılık gösterdiği belirlenmiştir. 14K02 karayemiş çekirdeğinin protein içeriği bakımından, 08K02 ve 61K04 karayemiş meyveleri ile kısmen benzerlik gösterdiği belirlenmiştir.

tablo 3'de belirtildiği üzere çalışmamızda karayemiş çekirdeklerine ait yağ oranları incelendiğinde 14K02 (Bolu) kod numaralı örneklerin yağ içeriği (%33,800) en fazla iken 28K13 (Giresun) kod numaralı çekirdeklerin yağ oranının (%25,773) en az olduğu görülmektedir. İstatistiki olarak incelendiğinde; 61K04 (Trabzon), 14K02 (Bolu) ve 08K02 (Artvin) karayemiş çekirdeklerinin yağ içerikleri yönünden birbirleri ile benzerlik gösterirken, 28K13 (Giresun) karayemiş çekirdeğinin yağ içeriği yönünden farklılık gösterdiği belirlenmiştir.

Tablo 4'de karayemiş çekirdeklerinin glukoz, fruktoz, sakkaroz ve diyet lifi içerikleri verilmiştir. Karayemiş çekirdekleri şeker bileşenlerinden glukoz ve fruktoz miktarları incelendiğinde en fazla glukoz ve fruktoz değerlerinin 61K04 (Trabzon) numaralı karayemiş çekirdeğinde, en düşük glukoz ve fruktoz değerlerinin ise 08K02 (Artvin) kod numaralı karayemiş çekirdeğinde bulunduğu görülmektedir.

Karayemiş çekirdekleri glukoz ve fruktoz değerleri açısından $p<0,05$ düzeyinde istatistiki olarak incelendiğinde; 61K04, 14K02, 28K13 ve 08K02 karayemiş çekirdeklerinin glukoz ve fruktoz içerikleri yönünden birbirleri ile farklılık gösterdiği belirlenmiştir.

Karayemiş çekirdek örneklerinde diyet lifi oranları incelendiğinde en fazla diyet lif miktarına 14K02 (Bolu) kod numaralı çekirdekte tespit edilirken en düşük değer 28K13 (Giresun) kod numaralı çekirdekte tespit edilmiştir. Karayemiş çekirdekleri diyet lifi değerleri istatistiki açıdan incelendiğinde; 61K04 (Trabzon), ve 28K13 (Giresun) karayemiş çekirdeklerinin diyet lifi miktarları yönünden birbirleri ile benzerlik gösterirken, 14K02 (Bolu) ve 08K02 (Artvin) karayemiş çekirdeklerinin diyet lifi miktarlarının birbirinden farklılık gösterdiği belirlenmiştir.

TARTIŞMA

Bu bulgular doğrultusunda farklı tip karayemiş meyveleri içerisinde en büyük meyveler ağırlık, en ve boy açısından 08K02 (Artvin) kodlu karayemiş tipinde, en küçük meyvelerin ise 14K02 (Bolu)kodlu tipinde olduğu tespit edilmiştir. İslam ve Deligöz (2012), Ordu ili merkez ve ilçelerinde 3000 Karayemiş ağacı gözlemleyip 82 adet Karayemiş tipi incelemişlerdir. Yaptıkları çalışmada tiplerinin meyve enini 11,95 mm ile 20,54 mm, meyve boyunu 12,15 mm ile 23,13 mm ve meyve ağırlıklarını 1,47 gr ile 6,24 gr arasında olduğunu belirlemişlerdir. Karan (2015) tarafından farklı karayemiş (*Prunus laurocerasus* L.) genotiplerinin depolama süresince kalite değişimlerinin incelendiği sekiz farklı tip karayemiş meyvelerinin enleri 14,27 mm-20,95 mm arasında, boyları ise 13,75 mm-19,21 mm arasında belirtilmiştir. Yine aynı çalışmada karayemiş meyvelerinin ağırlıkları ise 1,80 gr-4,93 gr arasında belirtilmiştir.

Tablo 3. Farklı tip karayemiş çekirdeklerine ait temel kompozisyonel analizler
Table 3. Basic compositional analyzes of different types of cherry laurel fruits seeds.

Analizler	61K04 (Trabzon)	14K02 (Bolu)	28K13 (Giresun)	08K02 (Artvin)
Kabuklu Ağırlık (gr)	0,130±0,015[a]	0,123±0,002[a]	0,153±0,023[a]	0,174±0,047[a]
İç Ağırlık (gr)	0,065±0,0127[b]	0,070±0,004[b]	0,081±0,013[b]	0,110±0,004[a]
Rutubet (%)	6,110±0,059[a]	6,301 ±0,083[a]	4,020 ±0,000[c]	4,630 ±0,000[b]
Kül (%)	3,400±0,031[b]	4,173±0,092[a]	2,722±0,009[c]	2,808±0,112[c]
pH	5,415±0,007[d]	5,630±0,000[c]	5,730±0,000[a]	5,660±0,000[b]
Protein (%)	25,156±0,076[b]	25,602±0,156[ab]	17,693±0,165[c]	26,051±0,117[a]
Yağ (%)	33,280±1,250[a]	33,800±1,500[a]	25,773±0,634[b]	33,227±1,202[a]

[a- d] Farklı harfle işaretlenmiş ortalamalar istatistiki olarak (p <0,05) biri birinden farklıdır. ±: Standart sapma.

Tablo 4. Farklı tip karayemiş çekirdekleri glukoz, fruktoz, sakkaroz ve diyet lifi içeriği
Table 4. Glucose, fructose, sucrose and dietary fiber content of different types of cherry laurel seeds

Karayemiş Çekirdek	Glukoz (gr.100gr^{-1})	Fruktoz (gr.100gr^{-1})	Sakkaroz (gr.100gr^{-1})	Diyet Lifi (%)
61K04 (Trabzon)	10,958±0,064[a]	2,581±0,047[a]	T.E.	16,671±0,023[c]
14K02 (Bolu)	9,335±0,027[b]	1,725±0,053[b]	T.E.	26,560±0,170[a]
28K13 (Giresun)	6,834±0,121[c]	1,341±0,054[c]	T.E.	16,505±0,002[c]
08K02 (Artvin)	4,841±0,117[d]	0,584±0,069[d]	T.E.	25,787±0,007[b]

[a- d] Farklı harfle işaretlenmiş ortalamalar istatistiki olarak (p <0,05) biri birinden farklıdır. ±: Standart sapma. T.E.: tespit edilemedi.

Karayemiş meyvesi açısından en önemli kalite kriterlerinden biri meyve ağırlığı, meyve eti/çekirdek oranı gibi parametrelerdir (İslam ve Deligöz 2012). Akbulut ve ark. (2007) tarafından, Karadeniz Bölgesinde 28 tip karayemiş meyveleri üzerinde yapılan çalışmada meyve ağırlığını 1,40-5,39 gr arasında, Yavuz (2018) tarafından farklı yenilebilir kaplamaların Karayemiş *(Prunus laurocerasus L.)* meyvesinin bazı kalite özellikleri üzerine etkisinin incelendiği diğer bir çalışmada ise, meyve ağırlığı 4,8801±0,9488 gr, meyve eni 21,1941±1,4099 mm ve meyve boyu ise 20,9500±1,4621 mm olarak belirtilmiştir. Araştırma sonuçlarımıza göre incelediğimiz karayemiş tiplerinin Karadeniz bölgesinde yapılan diğer çalışmalarda belirlenen en, boy ve ağırlık özelliklerinin benzerlik gösterdiği görülmektedir.

Sahan ve ark. (2010) yaptıkları çalışmada Düzce ilinden topladığı karayemiş meyvelerinin nem oranı'nı %79,48-80,19 belirlemişlerdir. Kolaylı ve ark. (2003) Trabzon bölgesinden temin edilen karayemiş meyvelerinin nem oranını %80 olarak belirlemişlerdir. Alasalvar ve ark. (2005) tarafından Giresun'da yetişen iki farklı çeşit karayemiş meyvesinin kimyasal ve antioksidan değerleri üzerine yapılan çalışmada nem (%) değerleri sırasıyla 81,21 ve 77,28 olarak belirtmişlerdir. Farklı karayemiş tiplerinde belirlediğimiz nem içerikleri bu çalışmalarda tespit edilen değerlerden daha düşük olarak belirlenmiştir.

Gıdalarda kül tayini ile organik maddelerin yanması ile geriye kalan inorganik kalıntı miktarı tespit edilir. Gıdalardaki kül içeriği toplam mineral içeriğini temsil eder (Harris ve Marshall 2017). Celik ve ark. (2011) yılında yaptıkları çalışmada incelenen karayemiş meyvelerinin kül oranı %0,25-%0,71 arasında değişen değerlerde tespit edilmiştir. Sakarya ilinde selekte edilen karayemiş (*Laurocerasus officinalis* R.) genotiplerinin antioksidan aktivite, fenolik bileşikler ve biyokimyasal özelliklerinin incelendiği bir çalışmada meyvenin kül miktarı %0,237 -0,720 olarak tespit edilmiştir (Beyhan ve ark.2018). Bir başka çalışmada ise karayemiş meyvelerinin tespit edilen kül oranları %0,67-%0,81 olarak belirtilmiştir (Sahan ve ark. 2010). Belirtilen çalışma sonuçları ile bizim araştırma sonuçlarımız kıyaslandığında 14K02 kod numaralı karayemiş meyvesinin kül oranı (%1,005)

dışında diğer karayemiş tiplerindeki kül değerleri benzer bulunmuştur.

pH değeri gıdalarda hidrojen iyon aktivitesinin bir ölçüsüdür ve dolayısı ile ürünün kalitesini belirleyen önemli bir unsurdur (Andrés-Bello ve ark. 2013). Çelik ve ark. (2011)'in, Doğu Karadeniz Bölgesi, Rize ilinde yetişen 11 adet karayemiş genotipinin fiziksel ve kimyasal meyve karakteristiklerini inceledikleri çalışmada pH değerleri 4,30-4,93 arasında bulunmuştur. Bir diğer çalışmada pH değerleri karayemiş meyve çeşitlerinde 4,8 olarak belirlenmiştir (İslam, 2002). Çalışmamızda incelediğimiz tüm karayemiş örneklerindeki pH değerleri bu değerlerin altında tespit edilmiştir.

Sahan ve ark. (2010) yaptıkları çalışmada Düzce ilinden topladığı karayemiş meyvelerinin protein oranlarını %0,92 - 0,99 belirlemişlerdir. Çelik ve ark. (2011) tarafından 11 farklı karayemiş genotipinin incelendiği çalışmada protein oranları %1,35-2,09 olarak tespit edilmiştir. Farklı bir çalışmada incelenen karayemiş varyetelerinin protein oranları (gr.100 gr^{-1}) 1,51 ve 0,54 olarak tespit edilmiştir (Alasalvar ve ark.(2005). Çalışmamızda tespit ettiğimiz değerler Alasalvar ve ark. (2005) tarafından protein oranı %0,54 olarak belirtilen karayemiş çeşidinden yüksek, Çelik ve ark. (2011)tarafından tespit edilen değerlerden düşük olarak bulunmuştur. 28K13 (Giresun) kod numaralı karayemiş meyveleri ise Sahan ve ark. (2010) tarafından yapılan çalışmada tespit edilen karayemiş meyveleri protein oranları ile benzer olarak tespit edilmiştir.

Sahan ve ark. (2010), yaptıkları çalışmada Düzce ilinden topladığı Karayemiş meyvelerinin yağ oranlarını %0,14-0,17 olarak, Alasalvar ve ark. (2005) tarafından yapılan çalışmada ise 0,23-0,10 gr.100 gr^{-1} olarak tespit edilmiştir. Çalışmamızda incelenen karayemiş meyvelerinde yağ tespit edilememiştir.

Giresun'da yetişen iki farklı varyetenin incelendiği, Alasalvar ve ark. (2005), tarafından yapılan çalışmada glukoz 5,88±0,42 gr.100gr^{-1} ve 5,43±0,21 gr.100gr^{-1}; fruktoz 5,16±0,51 gr.100gr^{-1} ve 4,84±0,19 gr.100gr^{-1} tespit edilmiştir. Çalışmamızda (gr.100 gr^{-1}) 4,541±0,175 ve 3,605±0,061 olarak tespit edilen 28K13 (Giresun) ve 08K02 (Artvin) kod numaralı karayemiş meyvelerinin fruktoz miktarları Alasalvar ve ark.(2005) tarafından yapılan çalışmada bulunan sonuçlardan düşük (gr.100 gr^{-1}); 6,620±0,052 ve 7,176±0,307 olarak belirlenen 61K04 (Trabzon) ve 14K02(Bolu) kod numaralı karayemiş tiplerinin fruktoz değerleri ise yüksek olarak tespit edilmiştir. Çalışmamızda belirlenen glukoz miktarlarının (gr.100 gr^{-1}) ise tüm tiplerde (61K04 (Trabzon) 12,155±0,064, 14K02 (Bolu) 11,467±0,714, 28K13 (Giresun) 11,839±0,088, 08K02 (Artvin) 9,077±0,109) daha fazla olduğu tespit edilmiştir. Sekiz farklı tip karayemişin şeker bileşiminin belirlendiği başka bir çalışmada glukoz içeriğinin 1,89-2,77 gr.100gr^{-1} yaş ağırlık, fruktoz içeriğinin ise 6,93-8,03 gr.100 gr^{-1} yaş ağırlık ve sakkarozun hiçbir tipte tespit edilmediği belirtilmiştir (Karan, 2015). Çalışmamızda belirlediğimiz sonuçlar glukoz içeriği açısından Karan (2015) tarafından belirtilen değerlerden yüksek, fruktoz içeriği açısından ise düşük olarak tespit edilmiştir.

Abraão ve ark. (2023) tarafından yapılan nesli tükenmekte olan fakat ekonomik, tarımsal ve sağlık üzerine etkilerinden dolayı iyi bir besin kaynağı olduğu düşünülen daha az bilinen Prunus türlerinden biri olan ve Portekiz Karayemişi olarak da bilinen *Prunus lusitanica L.* meyvelerinin besin bileşenlerinin tespiti amacıyla 2016–2019 yılları arasında meyvelerin farklı lokasyonlarda ve farklı yıllarda besin içeriklerinin incelendiği bir çalışmada *Prunus lusitanica L* meyvelerinin diyet lifi miktarı (g.100gr^{-1}) taze meyvede 2016 yılında 8,22-9,80 ve 10,26; 2017 yılında 8,76- 8,81-9,34; 2018 yılında 10,81-8,60-12.08 ve 2019 yılında 7,76-6,68-9,97 olarak belirtilmiştir. Bu değerler bizim çalışmamızda kuru meyvede tespit ettiğimiz değerlerden yüksektir. Ayrıca besin bileşenlerindeki farklılıkların çeşitli faktörlerin yanı sıra, yağış ve sıcaklık gibi çevresel faktörlerle ilişkili olduğu belirtilmiştir (Abraão ve ark. 2023).

Diyet lifleri sağlığın sürdürülmesi ve bazı hastalıklardan korunmada ve tedavisinde etkilidir (Samur ve Mercanlıgil 2008; Oğur 2019). Gıda sistemlerinin bir lif kaynağı olduğunu söyleyebilmek için 100 gr başına en az 3 gr lif veya 100 kcal başına en az 1,5 gr lif içermesi gerekmektedir (EC, 1924/2006; Abraão ve ark. 2023). Bu değerler karayemiş meyvesinin oldukça iyi bir diyet lifi kaynağı olabileceğini göstermektedir.

Macit ve Demirsoy (2012) tarafından Karayemiş (*Prunus laurocerasus L*) genotipleri ile ilgili yapılan bir çalışmada incelenen dört adet genotipin çekirdek ağırlıkları 0,31-0,56 gr arasında değiştiği belirtilmiştir. Karadeniz ve Kalkışım (1996), tarafından yapılan Akçaabat'da yetiştirilen karayemiş (*Prunus laurocerasus* L) tiplerindeki seleksiyon çalışmaları ile ilgili araştırmasında çekirdek ağırlıkları 0,17-0,75 gr arasında tespit edilmiştir. Çalışmamızda belirlediğimiz 08K02 (Artvin) kod numaralı çekirdek ağırlıkları Karadeniz ve Kalkışım (1996) tarafından yapılan çalışmadaki çekirdek ağırlıkları ile benzerlik gösterirken bunun haricindeki tüm karayemiş çekirdek ağırlıkları çalışmamızda belirlediğimiz değerlerden büyüktür.

Yapılan literatür araştırması sonucunda karayemiş çekirdeklerinin bazı fizikokimyasal özellikleri ile ilgili yeterli araştırmaya rastlanamamıştır. Bu sebeple bazı özellikleri *Rosaceae* familyasında bulunan ve karayemiş meyvesine renk, çekirdek şekli, çekirdek sayısı gibi fiziksel özellikleri ile benzerliği olan farklı meyvelerin çekirdek özellikleri ile kıyaslanmıştır.

Kayısı çekirdeklerinin pomolojik ve fizikokimyasal özelliklerinin karşılaştırıldığı farklı bir çalışmada ise kayısı çekirdek içlerinin nem değerleri %2,92-28,37 arasında bulunmuştur (Aydın, 2022). Yılmaz ve Gökmen (2013) tarafından vişne çekirdeklerinin nemi üzerine yapılan bir çalışmada 3,91±0,11 gr.100 gr^{-1} olarak çalışmamızda karayemiş meyve çekirdeklerine

ait bulduğumuz nem içeriği sonuçlarından daha düşük belirlenmiştir. Çalışmamızda belirlediğimiz nem değerleri, bu çalışmada belirlenen kayısı çekirdek içlerinin nem değerleri ile uyumludur.

Yılmaz ve Gökmen (2013) tarafından vişnede yapılan incelemelerde vişne çekirdeğinin kül değeri 3,11 ± 0,49 gr olarak belirtilmiştir. Aydın, (2022) tarafından kayısı çekirdeklerinde yapılan çalışmada ise kül ve pH değerlerinin %1,85-%3,42; 6,49-6,69 arasında bulunduğu belirtilmiştir. Vişne ve kayısı çekirdeklerinde belirlenen bu değerler bizim çalışmamızdaki kül değerleri açısından 14K02 (Bolu) kod numaralı çekirdek hariç diğer çekirdek örnekleri ile uyumlu, pH değerleri açısından ise tespit ettiğimiz sonuçlar literatür değerinin altındadır.

Yılmaz ve Gökmen (2013) tarafından yapılan çalışmada vişne çekirdeklerinde protein değeri 29,34±2,20 g, Aydın (2022), tarafından kayısı çeşitlerinde belirlenen protein değerleri ise %18,90±0,26 - %26,48±0,07 şeklindedir. Yaptığımız çalışmada incelediğimiz karayemiş tiplerinin çekirdeklerindeki protein oranları kayısı örneklerinde tespit edilen değerlerden 28K13 (Giresun) haricinde benzerdir. 28K13 kod numaralı karayemiş çekirdeği protein oranı belirtilen vişne ve kayısı çekirdeklerine ait değerlerden daha düşüktür. Vişne çekirdeğine ait protein oranı ise karayemiş çekirdeği protein oranından daha yüksektir.

Alasalvar ve ark. (2006) tarafından iki farklı karayemiş çekirdeklerinin yağ içerikleri % 38,10±0,32 ve %41,61±0.03 olarak belirlenmiştir. Karayemiş çekirdeklerinde yapılan başka bir çalışmada ise karayemiş çekirdeği yağ oranı %18,3 olarak tespit edilmiştir (Özgül-Yücel 2005). Araştırmamızda ki farklı tip karayemiş meyve çekirdeklerindeki yağ oranları Alasalvar ve ark. (2006) tarafından yapılan çalışmadaki sonuçlardan daha düşük, Özgül-Yücel (2005) tarafından yapılan çalışmadan daha yüksek olarak tespit edilmiştir.

Yılmaz ve Gökmen (2013) tarafından vişne çekirdeklerinde yapılan bir çalışmada çekirdeklerde 0.96±0.05 g glukoz, 1,65±0,25 g fruktoz ve 0,30±0,00 g sakaroz tespit edilmiştir. Karayemiş çekirdeklerinde tespit ettiğimiz glukoz ve fruktoz miktarları, Yılmaz ve Gökmen (2013) tarafından vişne çekirdeğinde tespit edilen bu değerlerden daha yüksektir. Karayemiş çekirdeklerinde sakkaroz tespit edilememiştir fakat vişne çekirdeğinde çok az da olsa sakkaroz bulunduğu bildirilmiştir.

García-Aguilar (2015) tarafından yapılan *Rosaceae* familyasına ait Kuzey Amerika, Guatemala, Colombia ve Venezuela gibi yerlerde yetişen *Prunus serotina Ehrh* (Amerikan siyah kirazı) meyvesi çekirdeklerinin besin değeri ve uçucu bileşikleri ile ilgili yapılan bir çalışmada kara kiraz meyvesinin çekirdeklerinin diyet lifi miktarının kuru maddede %10,73, bademde %10,91 ve yer fıstığında %9,21 olarak belirtilmiştir. Çalışmamızda tespit ettiğimiz farklı karayemiş tiplerinin diyet lif oranları bu değerlerden daha yüksek olarak belirlenmiştir. Karayemiş meyvelerinin kompozisyonlarındaki farklılıklar çeşit, hasat zamanı, mevsim, olgunluk, ağaçların yaşı, depolama özellikleri gibi unsurlardan kaynaklanabileceği belirtilmektedir (Ustun ve Tosun 2003, Alasalvar ve ark. 2005).

SONUÇ

Bu sonuçlar doğrultusunda farklı tipteki karayemiş meyvelernin ve çekirdeklerinin ağırlık, en, boy, rutubet, kül, pH, protein, fruktoz, glukoz ve diyet lifi içerikleri yönünden kendi aralarında tiplerine göre farklılık gösterdiği belirlenmiştir. Aynı parametreler açısından incelediğimiz farklı çalışma sonuçlarında dabazı parametrelerde farklılık tespit edilmiştir. Genel olarak farklı tip karayemiş meyve ve çekirdeklerinin belirtilen parametrelerin yanı sıra glukoz, fruktoz ve diyet lifi yönünden de fonksiyonel gıdalar bilimi için önemli meyvelerden birisi olabileceği belirlenmiştir. Özellikle çekirdek ile ilgili elde edilen bilgiler bu parametreler yönünden literatürdeki önemli boşluğu dolduracağı daha ileri düzeyde yapılacak olan çalışmalara ön çalışma olabilecek ve yeni bir bakış açısı kazandırabilecek nitelikte olduğu düşünülmektedir. Ayrıca, karayemiş çekirdeklerinin gıda olarak tüketilebilmesi için siyanojenik glikozitler açısından da incelenmesi tavsiye edilmektedir.

Çıkar çatışması: Yazarların rapor edecekleri herhangi bir çıkar çatışması yoktur.

Yazarların Katkıları: ÖET ve ED makaleye eşit katkıda bulunmuştur. ÖET ve ED, çalışmanın proje fikrine, tasarımına ve yürütülmesine katkıda bulundu. ÖET ve ED, verilerin toplanmasına katkıda bulundu. ÖET ve ED verileri analiz etti. ÖET ve ED taslağı hazırladı ve yazdı. ÖET ve ED taslağı eleştirel bir şekilde inceledi. Tüm yazarlar son halini alan makaleyi okudu ve onayladı.

Etik onay: Etik kurul sertifikasına gerek yoktur. Bu makalede sunulan veri, bilgi ve belgeler akademik ve etik kurallar çerçevesinde elde edilmiştir.

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RESEARCH ARTICLE

The Skull Shape Analysis of Wistar Albino Rats: A Geometric Morphometric Study

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ABSTRACT

The aim of the study was to evaluate the skull shape of *Wistar albino* rats using the geometric morphometry method. For this purpose, a total of 52 adult rat skulls, 31 female and 21 male, were evaluated. Skulls were photographed from equal distances in dorsal and ventral directions. 13 and 18 landmarks were marked in dorsal and ventral images, respectively. Percentage variation values of principal components were calculated by performing principal components analysis in the MorphoJ program. Average shape graphs showing the positive and negative boundaries of the first three principal components were obtained. It was defined that scatter plot showing the degree of grouping of male and female rats according to the first and second principal components. The study also examined the allometric effect with regression. Discriminant function analysis was performed to examine gender dimorphism. As a result of the study, it was determined that the most significant shape change was in the zygomatic arc and palate regions according to individuals, while it was also in the neurocranium according to gender. We believe that the study will contribute to anatomy, biology and archeology studies on the shape of the skull.

Keywords: Gender, Geometric Morphometry, Rat, Shape, Skull.

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Wistar Albino Ratların Kafatası Şekil Analizi: Bir Geometrik Morfometrik Çalışma

ÖZ

Çalışmada *Wistar albino* ratlarda kafatası şeklinin geometrik morfometri yöntemi ile değerlendirilmesi amaçlandı. Bu amaçla erişkin 31 dişi, 21 erkek olmak üzere toplam 52 adet rat kafatası değerlendirildi. Kafatasları dorsal ve ventral yönde eşit uzaklıktan fotoğraflandı. Dorsal görüntülerde 13, ventral görüntülerde 18 landmark işaretlendi. MorphoJ programında temel bileşenler analizi yapılarak temel bileşenlerin yüzde varyasyon değerleri hesaplandı ve ilk üç principal componentin pozitif negatif sınırlarını gösteren ortalama şekil grafikleri elde edildi. Erkek ve dişi ratların birinci ve ikinci temel bileşene göre gruplanma derecelerini gösteren dağılım grafiği tanımlandı. Çalışmada ayrıca regresyon ile allometrik etkiye bakıldı. Cinsiyet farkını incelemek için discriminant (ayırma) analizi yapıldı. Çalışma sonucunda wistar rat kafatasında bireyler arasında en belirgin şekilsel değişimin arcus zygomaticus (elmacık kemeri) ve damak bölgesinde olduğu, cinsiyete göre ise neurocranium da olduğu belirlendi. Çalışmanın, kafatasında cinsiyet farklılığı ile ilgili anatomi, biyoloji ve arkeoloji çalışmalarına katkı sağlayacağı kanaatindeyiz.

Anahtar Kelimeler: Cinsiyet, Geometrik Morfometri, Kafatası, Rat, Şekil

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INTRODUCTION

The *Wistar albino* rat is an outbred albino rat. This breed was developed at the Wistar Institute in 1906 for use in biological and medical research. It was the first rat to be developed as a model organism at a time when laboratories were primarily using the house mouse (Mus musculus). The Wistar rat is currently one of the most popular rats used in laboratory research. It is characterized by its broad head, long ears, and tail length that is always shorter than its body length (Clause 1998).

One of the structures frequently used in morphometric research on the skeletal system is the skull. The bones that make up the skull are in the flat bones group. Anatomically, the skull is examined in two parts: viscerocranium (facial bones) and neurocranium (cranial bones) (Özcan and Demiraslan 2022). The morphological and morphometrical structure of these bones differs according to species and gender (Çalışır et al. 2023). For this reason, comparative morphometric or geometric morphometric shape analyses on the skull are a real tool in the classification of rats and identification of species (Musser et al. 2005; Odigie et al. 2018).

The geometric morphometry method is a field of study that investigates and analyzes the variations and changes seen in structural (morphological) features by using landmarks on the surface area that is examined. It allows the comparison and interpretation of structural differences owing to visual graphs obtained with Cartesian coordinates (Slice 2007; Slice et al. 2010; Mitteroecker and Gunz 2009).

Statistically significant differences were determined in the skull related to structural changes due to bone development and function (Reichs and Bass 1998). Research on gender dimorphism in the skull is frequently carried out using the morphometry method (Karaavcı et al. 2024; Gündemir et al. 2023a, 2023b; Dayan et al. 2023; Szara et al. 2022; Gürbüz et al. 2022; Demircioğlu et al. 2021). Researches have previously been carried out in rat skulls with morphometric methods based on the linear measurement method (Olude et al. 2009). However, no study was found that evaluated the structural properties of the skull in *Wistar albino* rats using the geometric morphometric method. In this study, it was aimed to determine the regions where there are structural differences and similarities in the skull of *Wistar albino rats* and to compare them according to the gender factor.

MATERIALS and METHODS

Animals Ethical Approval

The necessary permission for the study was obtained from Burdur Mehmet Akif Ersoy University, Animal Experiments Local Ethics Committee (Date: 29.03.2023, No:996).

Materials

A total of 52 rat (Wistar albino) skulls, 31 female and 21 male, were used in the study. The rats used in the study were chosen from those raised in similar care units and were adult and healthy. Analysis began after the skulls were cleaned of their buttocks by maceration.

Geometric Morphometry

Rat skulls were photographed from equal distances, dorsal and ventral view. In the photographed images, 13 and 18 homologous landmark points were detected from the dorsal direction (Figure 1) and ventral direction (Figure 2), respectively. TPS files of the images were created using the TPSUtil (version 1.79) program (Rohlf 2019). Homologous landmarks detected in TPS Dig2 (Version 2.31) (Rohlf 2018). The program were marked. Thus, the x and y Cartesian coordinates of the homologous landmarks on the skull were determined. The suitability of landmarks was tested in TPS small (version 1.34) program and their correct placement was proven. In dorsal images the slope and correlation score were determined as 0.99820 and 1.0000 respectively; in ventral images, the slope and correlation score were determined as 0.999895 and 1.0000.

Statistical Analysis

MorphoJ software was used in the study for statistical analysis and evaluation of shape differences (Klingenberg, 2011). In this program, firstly, Generalized Procrustes Analysis (GPA) was performed to eliminate differences in skull placement and position in the photographs (Slice 2007). Thus, the landmarks on images were superimposed, new coordinates were determined and Principal Component Analysis (PCA) was performed. Degrees of interindividual variation were determined as percentage (%) values (Zelditch et al. 2012; Villalobos-Leiva and Benitez 2020). To determine whether shape changes in the skull depending on size, regression and allometric effects were examined. Additionally, Discriminant Function Analysis was performed to determine the differences in skull shape according to gender factors (Zelditch et al. 2012).

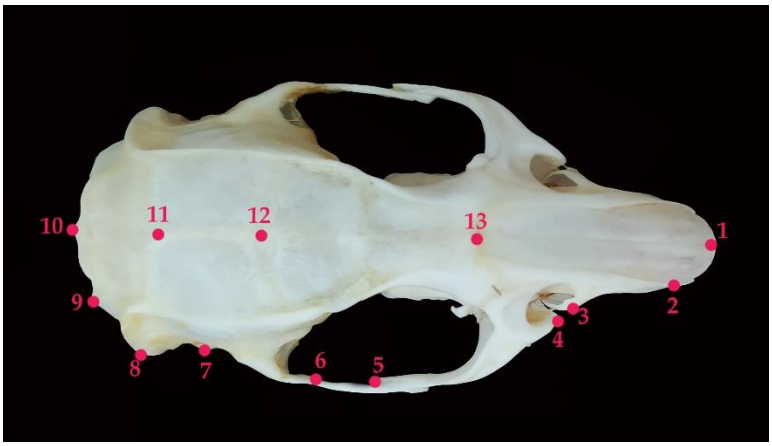

Figure 1. The landmarks on the dorsal view of rat skull

1. Cranial point of incisive bone (os incisivum), 2. Craniolateral point of nasal bone (os nasale), 3. The most lateral point of maxilla, 4. The most cranial point of zygomatic arc (arcus zygomaticus), 5. At the caudal end of maxillar process (processus maxillaris) of zygomatic arc (arcus zygomaticus), 6. At the cranial end of temporal process (processus temporalis) of zygomatic arc, 7. External meatus acusticus (meatus acusticus externus), 8. The lateral point of temporal bone (os temporale), 9. The most caudolateral point of occipital bone (os occipitale), 10. Caudomedial point of occipital bone, 11. The stura between occipitale and parietal bone (os parietale) (Lambda), 12. The sutura between parietal and frontal bone (os frontale) (Bregma), 13. The sutura between frontal and nasal bone.

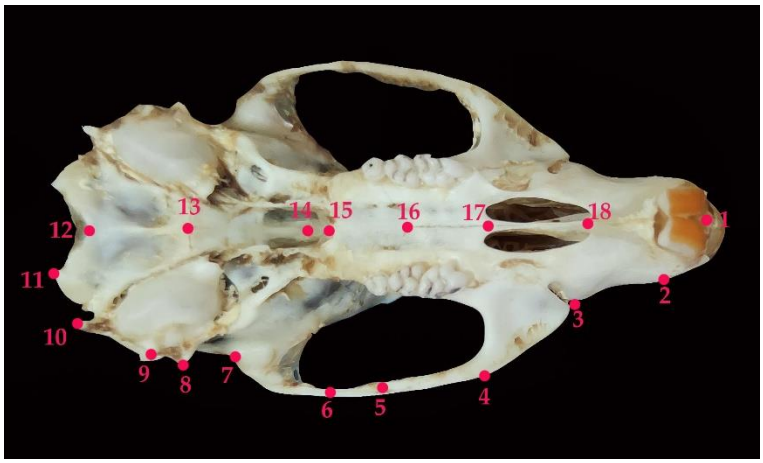

Figure 2. The landmarks on the ventral view of the rat skull

1. Cranial point of incisive bone, **2.** The most craniolateral point of maxilla, **3.** Lateral point of infraorbital foramen (*foramen infraorbitale*), **4.** The most craniolateral point of zygomatic arc, **5.** At the caudal end of maxillar process of zygomatic arc, **6.** At the cranial end point of temporal process of zygomatic arc, **7.** Caudal angle of zygomatic arc, **8.** Cranial point of external meatus acusticus, **9.** Caudal point of external meatus acusticus, **10.** Jugular pocess (*processus jugularis*), **11.** Caudal point of occipital condyle (*condylus occipitalis*), **12.** Ventral point of foramen magnum, **13.** Sutura between occipital (*os occipitale*) and sphenoid (*os sphenoidale*) bone, **14.** Medial point of sutura between sphenoid and vomer bone, **15.** Caudomedial point of palatina (*os palatinum*), **16.** At the medial sutura between palatinum and maxilla, **17.** Craniomedial point of palatine process (*processus palatinus*) of maxilla, **18.** Caudomedial point of incisive bone

RESULTS

In the study, according to the principal component analysis performed in the dorsal direction, a total of 22 principal components were calculated and 32 principal components were calculated in the ventral images. In dorsal oriented images, the first principal component had a degree of variation of 19.431%, and in ventrally oriented images it had a degree of variation of 20.758%. In the dorsal and ventral images, the degree of variation in the first three principal components was determined as a total of 48.923% and 47.052%, respectively. The percentage variation values of the sequential principal components were determined to be close to each other (Table 1, Table 2).

Table 1. Principal component analysis on dorsal view of rat skulls

PC	Eigenvalues	%Variance	PC	Eigenvalues	%Variance
1	0.00016825	19.431	12	0.00001836	2.121
2	0.00014692	16.969	13	0.00001617	1.868
3	0.00010843	12.523	14	0.00001298	1.499
4	0.00007522	8.688	15	0.00001022	1.180
5	0.00007383	8.527	16	0.00000746	0.861
6	0.00005130	5.925	17	0.00000541	0.624
7	0.00004249	4.908	18	0.00000472	0.545
8	0.00003932	4.541	19	0.00000355	0.409
9	0.00002922	3.374	20	0.00000250	0.288
10	0.00002586	2.987	21	0.00000202	0.233
11	0.00001995	2.304	22	0.00000168	0.194

Table 2. Principal component analysis on ventral view of rat skulls

PC	Eigenvalues	%Variance	PC	Eigenvalues	%Variance
1	0.00018902	20.758	17	0.00001031	1.132
2	0.00014718	16.163	18	0.00000961	1.055
3	0.00009225	10.131	19	0.00000843	0.926
4	0.00008292	9.107	20	0.00000481	0.528
5	0.00006442	7.075	21	0.00000444	0.487
6	0.00005182	5.691	22	0.00000368	0.404
7	0.00004343	4.769	23	0.00000294	0.323
8	0.00003264	3.585	24	0.00000286	0.314
9	0.00002875	3.157	25	0.00000211	0.232
10	0.00002711	2.977	26	0.00000189	0.207
11	0.00002236	2.455	27	0.00000158	0.174
12	0.00002096	2.302	28	0.00000135	0.149
13	0.00001465	1.608	29	0.00000097	0.107
14	0.00001424	1.564	30	0.00000049	0.053
15	0.00001209	1.328	31	0.00000045	0.049
16	0.00001044	1.147	32	0.00000040	0.044

As a result of principal component analysis, PC1, PC2 and PC3 graphics were presented in Figure 3. It was observed that the most change was at Landmark 5, 6, 7, 12, and 13 levels in dorsal images and at Landmark 1, 2, 3, 4, 5, 6, and 7 levels in ventral images. It was determined that the greatest change in ventral and dorsal images was at the arcus zygomaticus level. However, it was determined that the change was mostly on neurocranium in dorsal images, but also mostly on viscerocranium in ventral images. The change in positive-negative boundaries was greater in ventral images than in dorsal images.

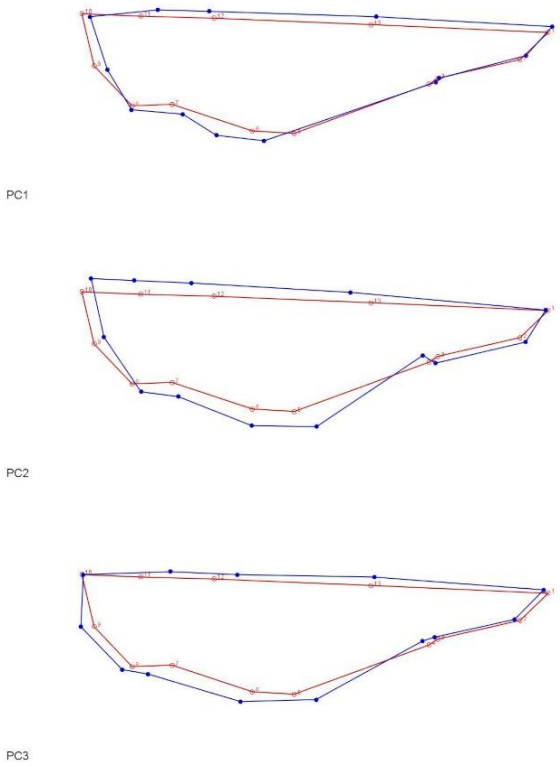

Figure 3. Shape changes in dorsal images of the skull relative to PC1, PC2 and PC3 (Blue outlines represent the average shape configuration, while red outlines indicate shape changes associated with the positive ends of the PC axes)

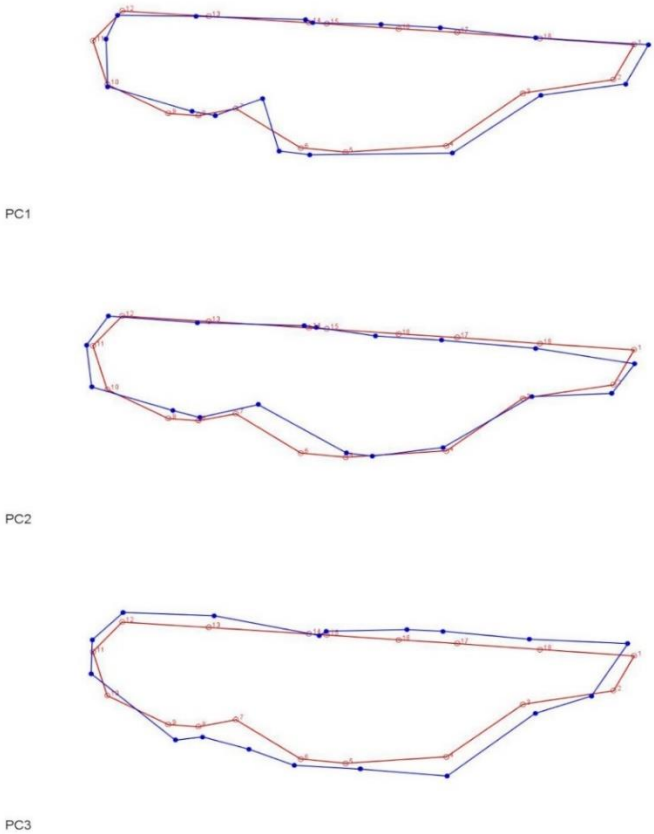

Figure 4. Shape changes in ventral images of the skull according to PC1, PC2 and PC3 (Blue outlines represent the average shape configuration, while red outlines indicate shape changes associated with the positive ends of the PC axes)

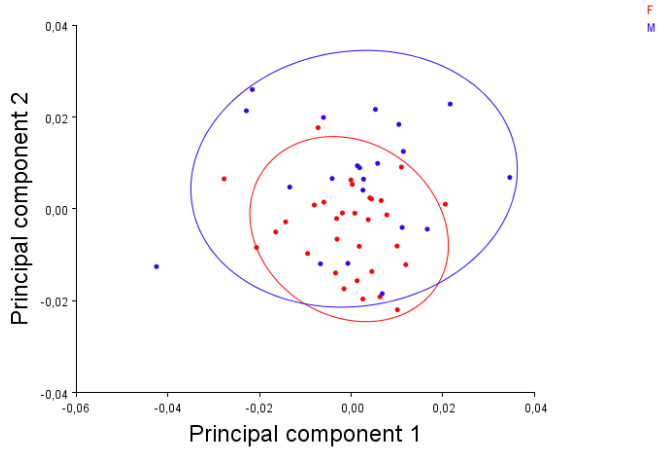

Figure 5. Scatter plot on the principal component axis 1 and principal component axis 2 for sexes on the dorsal images (Blue: male, Red: female).

In Figure 5 and Figure 6, distribution graphs of the skulls in dorsal and ventral images were presented. Scatter plots showed the degree to which skulls were grouped by gender. Accordingly, it was seen that the male and female skulls were not clearly separated from each other.

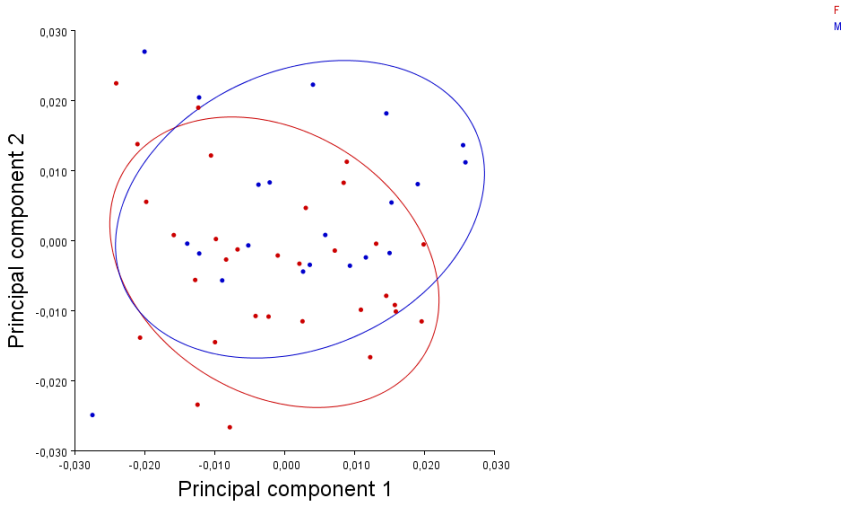

Figure 6. Scatter plot on the principal component axis1 and principal component axis 2 for sexes on the ventral images (Blue: male, Red: female).

Before discriminant function analysis, the allometric effect was examined by regression to evaluate whether the size effected on the shape. The values are presented in the Table 3. Accordingly, the effect of size on shape was statistically significant ($p<0.05$). This effect was removed from the data set with multivariate analysis (Residuels/prediction from regression) and then the analysis continued.

Table 3. Regression analysis for sexual dimorphism comparison in centroid size, in both ventral and dorsal views of rat skulls.

Skull view	Group (n)	%Predicted	p value
Dorsal	Males (21), females (31)	5.2372	0.0033
Ventral	Males (21), females (31)	3.9063	0.0289

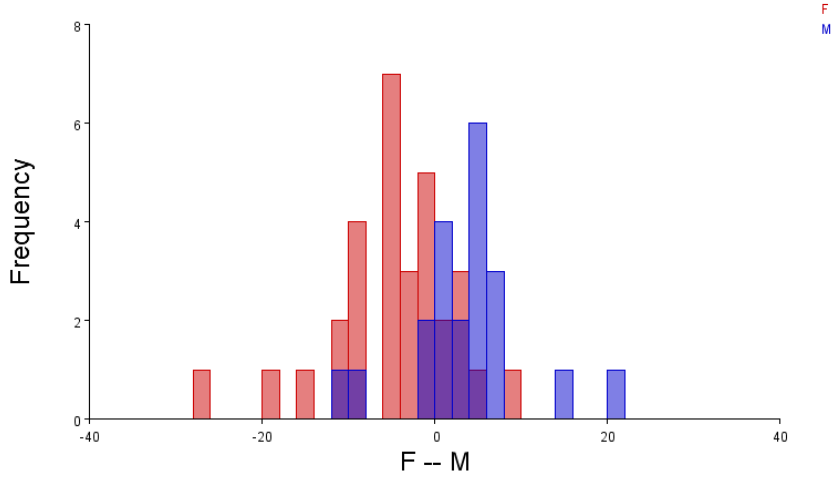

Figure 7. Cross validation score of discriminant function analysis on the dorsal view (Red: Female rat skull, Blue male rat skull).

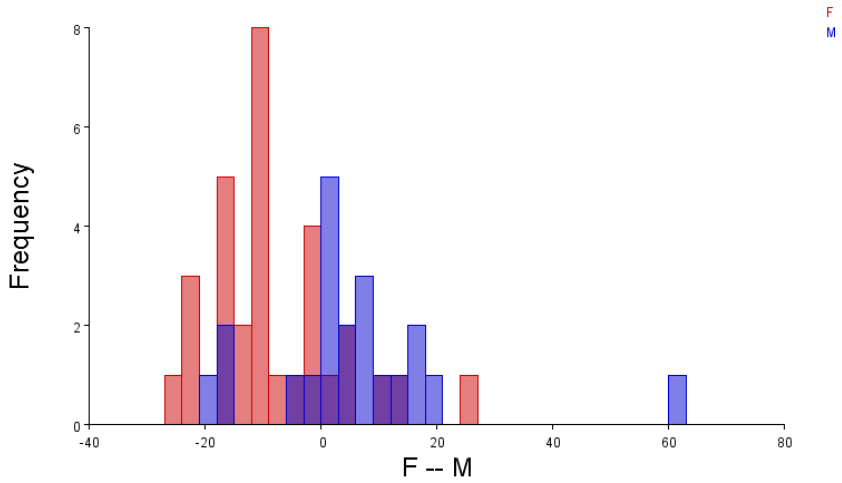

Figure 8. Cross validation score of discriminant function analysis on the ventral view (Red: Female rat skull, Blue male rat skull).

The cross-validation score determined by discriminant function analysis was shown in the graph in Figures 7 and 8. These graphs show the distribution of male and female skulls. According to this analysis, it is seen that 17 skulls from males and 24 skulls from females were grouped correctly in dorsal images, and 16 skulls from males and 25 skulls from females were grouped correctly in ventral images. As a result of dorsal analysis, Procrustes and Mahalanobis distance were determined as 0.0154 ($p<0.0001$) and 3.2274, respectively, and in ventral images, these values were determined as 0.0173 ($p<0.0001$) and 4.4596, respectively. The average shape obtained as a result of discriminant function analysis is shown in Figures 9 and 10. In this figure, the general shape of the skull of male and female rats was similar. However, when looking at the average shape of males and females in dorsal and ventral images, small differences were observed in the neurocranium.

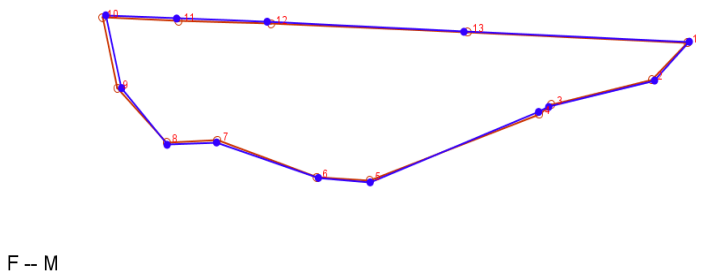

Figure 9. Discriminant function analysis on the dorsal view. (Red: female rat skull, Blue: male rat skull).

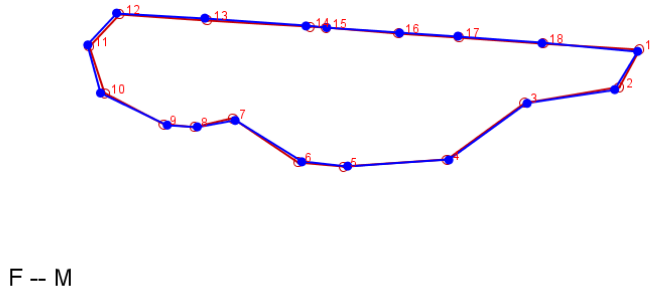

Figure 10. Discriminant function analysis on the ventral view. (Red: female rat skull, Blue: male rat skull)

DISCUSSION

In studies conducted on the skulls of rodents from different species, it has been reported that the structural differences observed on the cranium may be due to nutritional differences (Samuel et al. 2015). For example, African giant rats have been reported to be fed with household waste and less fibrous foods, and *Thryonomys (T.) swinderianus* species rodents have been reported to be fed with roughage and foods with high fiber content. It has been stated that this situation may cause changes in the anatomical parameters of the skull (Samuel et. al., 2015). Samuel et al. (2015), in a study conducted on the skulls of two species of African rodents, reported that although both rodents had similar tooth and jaw structures, the maxillo-dental structure of *T. swinderianus* was longer than that of *Cricetomys gambianus*. Researchers (Samuel et al. 2015) stated that partial shape changes in the bone structure are observed with strong horizontal movements of the head during chewing, and therefore the chewing movement causes specific changes in the morphological structure of the skull and jaw. In the study, it is thought that the shape difference detected in the zygomatic arc region where the chewing muscles attach, and in the palate region may be due to the chewing movement (biting force). Identification of the characters that constitute cranial variation is important for determining population history, adaptation, and genetic structure (Lieberman et al. 2000; Abdel-Rahman et al. 2009; Herrel et al. 2012). Differences in the shape and size of the skull allow understanding of its development from embryo to adult (Herrel et al. 2012). During the developmental process, the morphological structure of the cranium varies according to age, genetic and environmental factors. One of these sources of phenotypic variation is gender difference (Gannon and Racz 2006). Phenotypic variation based on gender differences is important for animal behavior, ecology, generation mobility, and evolution (Leblanc et al. 2001). Shape differences can be detected according to gender factor with morphometric methods. In a morphometric study conducted with linear measurements on Black-hooded rats (Hughes et al. 1978), it was determined that skull measurements in male rats were significantly bigger than in females, but there was no difference between genders in cranial and facial indices. Çalışır et al. (2023) reported that the craniofacial index value was statistically significant according to the breed factors in male rats. However, Çalışır et al. (2023) stated that there is no difference in cranial and facial indices according to gender. In this study, when compared by

gender using discriminant function analysis, small differences were observed in the neurocranium, while no significant difference was observed in the viscerocranium.

Another structure that affects shape changes in the skull is the mechanical role of cranial sutures (Sharp et al. 2023). In a study on the role of cranial suture's in the rat skull (Sharp et al. 2023), it was reported that cranial sutures in regions such as the temporomandibular joint or maxilla may be more significantly affected than other regions during feeding. However, it has been emphasized that these sutures surrounding the brain (neurocranium) may be important in allowing the brain to expand rather than biting power during growth. In this study, it was determined that the structural variation of the skull between individuals was observed mostly in the part where the chewing muscles attach (zygomatic arc, palate region), while the variation according to gender was found in the neurocranium. While this variation determined between individuals is more related to chewing function, it is thought that the variation determined by gender may be more related to the genetic and developmental processes.

CONCLUSION

As a result, in the inter-individual comparison, the shape changes were evident in the functional areas to which the chewing muscles attach, especially on the zygomatic arc region and the palate region. In this study, we also tried to determine the effect of gender factors on skull shape in rats. In the gender comparison, although male and female skull shapes were observed to be generally similar, minor shape changes were observed on the neurocranium. Accordingly, it was determined that the neurocranium of male rats in dorsal images and female rats in ventral images was slightly narrower. As a result of the evaluation made according to the grouping characteristics, it was determined that 80% of the males in the dorsal images and 80% of the females in the ventral images were grouped correctly. When all the findings of the research were evaluated, it was concluded that despite the differences between the genders, these features were not sufficient to distinguish the two genders from each other.

Conflict of Interest: The authors have no conflicts of interest to report.

Authors' Contributions: İG and YD contributed to the project idea, design, and execution of the study. İG contributed to the acquisition of data. İG analyzed the data. İG drafted and wrote the manuscript. YD reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical Approval: This study was carried out at Burdur Mehmet Akif Ersoy University, Animal Experiments Local Ethics Committee (Date: 29.03.2023, No:996).

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RESEARCH ARTICLE

The Effects of Colloidal Silver Solution and Hypochlorous Acid on Wound Healing in the New Zealand Rabbit

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ABSTRACT

The aim of this study was to investigate and evaluate the effects of colloidal silver and hypochlorous acid solutions on wound healing. To evaluate the wound healing activity of colloidal silver solution and hypochlorous acid solutions, an in vivo excisional wound model was applied in New Zealand rabbits (n = 21). The rabbits were randomly divided into 3 groups with seven individuals in each group. Group 1 was used as a negative control and no treatment was applied. Groups 2 and 3 were treated with crystalline (hypochlorous acid) and colloidal silver, respectively. The treatments were applied topically (in the form of spray) to the wound area of each rabbit daily. Wound diameters were measured on days 4, 8 and 12 using calipers and histopathological examinations were performed on days 4, 8 and 12 of the treatment. In terms of wound closure, both hypochlorous acid and colloidal silver solutions showed comparable wound healing activity with the control group. In conclusion, hypochlorous acid and colloidal silver have a positive effect on dermal wound healing in rabbits.

Key Words: Rabbit, Hypochlorous acid, Colloidal silver, Wound healing

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Yeni Zelanda Tavşanında Kolloidal Gümüş Solüsyonu ve Hipokloröz Asitin Yara İyileşmesi Üzerine Etkileri

ÖZ

Bu çalışmanın amacı, kolloidal gümüş ve hipokloröz asit çözeltilerinin yara iyileşmesi üzerine etkilerinin araştırmak ve değerlendirmektir. Kolloidal gümüş çözeltisi ve hipokloröz asit solüsyonlarının yara iyileştirme aktivitesini değerlendirmek için Yeni Zelanda tavşanlarında (n = 21) in vivo ekzisyonel yara modeli uygulandı. Tavşanlar, her grupta yedi bireyden oluşacak şekilde rastgele 3 gruba ayrıldı. Grup 1 negatif kontrol olarak kullanıldı ve hiçbir tedavi uygulanmadı. Grup 2 ve 3 sırasıyla kristalin (hipokloröz asit) ve kolloidal gümüş ile tedavi edildi. Tedaviler yara bölgesine topikal olarak (sprey şeklinde), her tavşanın yara bölgesine günlük olarak uygulandı. Yara çapları 4, 8 ve 12. günlerde kumpas kullanılarak ölçülmüş ve tedavinin yine 4, 8 ve 12. günlerinde histopatolojik incelemeler yapılmıştır. Yara kapanması açısından, hem hipokloröz asit hem de kolloidal gümüş solüsyonları kontrol grubu ile karşılaştırılabilir yara iyileştirme aktivitesi göstermiştir. Sonuç olarak, hipokloröz asit ve kolloidal gümüş tavşanlarda dermal yara iyileşmesi üzerinde olumlu bir etki göstermektedir..

Anahtar Kelimeler: Tavşan, Hipokloröz asit, Kolloidal gümüş, Yara iyileşme

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INTRODUCTION

Wound is a condition in which the integrity of the multilayered flat epithelium is disrupted and loses its function due to various reasons (Robson et al, 2001). The wound may be superficial or deep enough to cover tendon, muscle, vessel, nerve, bone, and subcutaneous connective tissue (Alonso et al, 1996). Wound healing is a synchronised and complicated biological process that starts because of stimulation of many interconnected mechanisms (Gurtner et al, 2008). Wound healing stages generally consist of haemostasis, inflammation, cell proliferation and remodelling (Diegelmann and Evans, 2004). Injury can occur in all tissues and organs of the body and the healing process is similar in almost all of them. The healing process occurs with the cooperation of biological and immunological systems (Attinger et al, 2006). Wound products have a very important place in wound research and applications all over the world. These wound care products contribute to the natural healing process by protecting the tissue and activating cell production (Valenta et al, 2004).

Silver is a metal that has been encountered with different usage areas since ancient times (Serenella et al., 2019). In addition to wound dressings and creams in the medical field, silver is used in medical devices and in the coating of antibiotics (Jean-Yves et al., 2013). There are conclusive studies that the use of silver alloy indwelling catheters for short-term catheterisation will reduce the risk of catheter-induced urinary tract infections (Beattie M., 2011; Schumm et al., 2008). Silver generally has low toxicity and minimal risk when used in silver-approved medical applications (Lansdown, 2006). Until the invention of antibiotics, silver was used both for food preservation and as a good therapeutic agent. Today, silver is known to have antibacterial effect on nearly six hundred bacteria (Fung et al., 1996). Nanoparticle silver is obtained by separating silver metal into very small pieces in the order of nanometre. In this way, silver nanoparticles can be transported to very remote points in the living body through fluids. Silver has antibacterial effect as well as antiallergic effect. Ionic silver is formed by introducing silver into a liquid in the form of Ag + ions. This liquid is called colloidal in the sense of suspended. Since colloidal ionic silver atoms are positively charged, they are suspended in the liquid by repelling each other. This liquid can be applied to the skin or administered sublingually, by drinking or through the rectum to ensure absorption. Dose and concentration studies for intravenous applications are ongoing (Rosenblatt et al., 2009; Deery, 2009; Lancaster and Steady, 2010; Wesley, 2009). Silver and most silver compounds have oligodynamic effects and are toxic to bacteria, algae and fungi when used in vitro (Lansdown, 2006). The activity of silver compounds as antiseptics is such that the biologically active silver ion (Ag+) irreversibly damages key enzyme systems in the cell membranes of pathogens (Lansdown, 2006).

Hypochlorous acid (HOCl) is a non-cytotoxic microbiocidal agent active against all bacterial, viral, and fungal human pathogens (Mekkawy and Kamal, 2014). HOCl can be formed by combining acidic chlorine oxide (Cl) with water. Stabilised in the form of a physiologically stable solution, HOCl is a naturally occurring molecule produced by neutrophils to destroy pathogens (Gold et al., 2019). It has an excellent bactericidal effect against various microorganisms due to its high oxidising capacity and is highly advantageous due to its practical applicability in healthcare institutions or food industry (Tsai et al., 2023).

The main features of hypochlorous acid in its mechanism of action are oxidation of sulfhydryl enzymes, oxidation of amino acids, chlorination of amino acids, inhibition of protein synthesis, loss of intercellular substance, decrease in nutrient uptake, decrease in oxygen uptake, oxidation of respiratory components, decrease in adenosine triphosphate production, DNA breakage and suppression of DNA synthesis. It causes rapid death of all bacteria, viruses and fungi and inactivation of prions. Due to these properties, resistance cannot be developed by microorganisms (Sakarya, 2019).

This study aims to investigate the effects of colloidal silver and hypochlorous acid solutions on wound healing.

MATERIALS and METHODS

Wound Healing Activity

Animals

In this study, 21 male New Zealand rabbits with an average body weight of 2000-2500 g, approximately 6-12 months of age, were selected to create the excision wound model. The care, feeding and biopsy procedures of the animals to be used in the study were supervised at Burdur Mehmet Akif Ersoy University Experimental Animal Production and Experimental Research Centre. Rabbits were housed individually in standard cages measuring 45×40×50 cm. The rabbits were kept under standard conditions including 12:12 h light-dark cycle, 50-70% humidity and 25°C ± 3°C temperature range. In terms of feeding, they were given unlimited access to standard feed and water. The study was conducted in accordance with the guidelines of the European Community Council Directive (2010/63/EU). Burdur Mehmet Akif Ersoy University Rectorate Animal Experiments Local Ethics Committee approved the study as stated in its decision dated 15 January 2020 and numbered 613.

Excision Wound Model

The excision wound model procedure in rabbits was adapted from the protocol established by Huang et al. (2019). Rabbits were anaesthetised using a combination of xylazine and ketamine (3-5 mg/kg xylazine and 50 mg/kg ketamine). While anaesthetised, the dorsal skin of each rabbit was shaved using an electric razor and then disinfected with topical povidone-iodine. Four full-thickness wounds, each 8 mm in diameter, were made on both the left and right side of the midline on the back of each animal.

The wounds created are not immobilized in any way. The best model of wounds that would normally occur in nature are those that are not immobilized. However, fixation of the wounds has advantages such as supporting the healing process, faster healing, and reducing the risk of infection. In fixed wounds with well-united epithelialization edges, healing begins almost immediately as there is no defect to be filled by granulation tissue. Such reasons may also support the use of the immobilized wound model.

Considering the healing phases of the wound, the days determined for the wounds were selected and evaluated. Since the repair phase usually starts 3 to 5 days after the injury, we chose day 4 as the first day to be evaluated for the wound. Wound contraction reduces wound size through cells in the granulation tissue. Wound contraction is associated with a complex interaction of extracellular matrix, cells and cytokines. Due to such factors, the full-thickness skin edges of the wound, from the periphery to the center, are pulled inward by contraction. This shrinks noticeably within 5 to 9 days after injury. Taking this timing into account, we chose day 8 as the second day to evaluate the wound. During the maturation phase of the wound, the strength of the wound is maximized due to changes in the scar as the wound heals. The most rapid gain in wound strength occurs between 7 and 14 days after injury, when collagen rapidly accumulates in the wound. Taking this into account, we chose day 12 as the third day to evaluate the wound.

Three biopsies were taken from three different wounds in each animal. At the end of the study, the fourth wound was left unbiopsied to assess the degree of wound closure.

Treatment Schedule

Rabbits were randomly divided into 3 groups with seven individuals in each group. Group 1 was used as a negative control and no treatment was applied. Groups 2 and 3 were treated with crystalline (hypochlorous acid) and colloidal silver, respectively. The treatments were applied topically (in the form of a spray) to the wound area of each rabbit daily. Biopsies for histopathological evaluation were taken on days 4, 8 and 12 under xylazine and ketamine induced anaesthesia. The complete healing of the right caudal wound facilitated the assessment of the level of wound closure. On day 12, the diameters of the right caudal wounds were measured using callipers. Wound areas were also determined with a caliper immediately after wound induction. The quantitative data and results obtained from this study were primarily based on caliper measurements, ensuring the accuracy and reliability of our findings.

Histopathology

After fixation in 10% neutral buffered formaldehyde solution, 4 μm thick sections were taken from the biopsy specimens. The sections were embedded in paraffin and stained with haematoxylin and eosin (H & E). Histopathologists evaluated inflammatory cell infiltration (both acute and chronic), angiogenesis, fibroblast maturation levels and the amount of granulation tissue in the injured area. Angiogenesis was scored as 0=absent, 1=mild (less than 5 vessels at 1 high magnification), 2=moderate (6-10 vessels at 1 high magnification) and 3=significant (more than 10 vessels at 1 high magnification). Inflammation was scored as 1=very mild (0-25 cells at 3 high magnification), 2=mild (25-50 cells at 3 high magnification), 3=moderate (50-75 cells at 3 high magnification) and 4=significant (75-100 cells at 3 high magnification) and evaluated according to this scoring. The amount of fibrosis and fibroblast maturation levels were also scored as 0=absent, 1=mild, 2=moderate and 3=significant. The scoring approach was consistent with previous literature (Gül Satar et al., 2017; Güzel et al., 2019). Fibroblast maturation was evaluated according to cellular morphology and arrangement in the tissue. Mature fibroblasts were identified by their elongated spindle shape, arranged in parallel arrays, and reduced cellular density compared to more proliferative regions of granulation tissue. In addition, the presence of a well-developed extracellular matrix was indicative of active collagen production and deposition by mature fibroblasts. This served as an important criterion for assessing fibroblast maturity (Broughton et al., 2006). Group comparisons were made separately for each biopsy timeline.

Statistics

Whether the statistical analysis methods met the parametric test assumptions was checked by Kolmogorov-Smirnov test. Chi-square analysis method was used for comparison of all groups. One-way analysis of variance (ANOVA) or Friedman test was used for the comparison of related groups, and one-way analysis of variance (ANOVA) and Tukey test or Kruskal Wallis-H test was used for the comparison of independent groups. A significance level of $p<0.05$ was accepted. IBM SPSS Statistics 22 package programme was used for the test.Macroscopic Observations

Wound diameters were measured macroscopically, and wound areas were calculated. The obtained data were evaluated statistically on the 4th, 8th, and 12th days. On days 4 and 8, a statistically significant

difference was found in all 3 groups. On day 4, the colloidal silver group had the best wound healing ($p = 0.012$), followed by crystalline and control groups ($p < 0.001$ in both groups). On the 12th day, there was no statistical difference between the crystalline and colloidal silver groups ($p= 0,910$), while these two groups were statistically different from the control group ($p< 0,001$) and the healing rate was found to be better than the control group. Data of wound diameters after closure of wounds were given in Table 1.

Histopathological Findings

Biopsy specimens (8 mm in diameter) taken from different edges of the wounds of each rabbit on days 4, 8 and 12 with a punch biopsy tool were subjected to routine follow-up procedures for histopathological examinations after 10% formaldehyde fixation. Sections (5 μm) were stained with haematoxylin and eosin and evaluated under light microscope. Inflammatory cell counts and angiogenesis were evaluated to determine the inflammation and healing process during the healing phase of wound healing. For this purpose, inflammatory cells and vessels were counted in four high magnification fields (400x) from random areas in the epidermis or dermis under the crust from each skin section. Inflammatory cells were scored between 1-4 (0-25=1, 26-50=2, 51-75=3, >75=4). The same selected areas were analysed for the number of vessels and the numbers were noted. All histological sections were blindly evaluated by the same investigator. Minitab® 16.1.1 package programme was used to analyse the data obtained. After determining the normal distribution of the data by Ryan-Joiner normality test, One-Way Anova Tukey test was used to reveal the differences between the groups.

As a result of statistical analysis, a significant increase in angiogenesis was determined in the crystalline group on the 4th day compared to the control and silver groups. On the 8th and 12th days, no significant difference was observed between the groups. In terms of inflammatory cell score, there was no significant difference between the groups on the 4th and 8th days, while a significant increase was observed in the crystallin group compared to the silver group on the 12th day, but no significant difference was found between both groups and the control group (Figure 1). Statistical data were given in Table 2. In the light of these data, although crystallin application increases angiogenesis in the early period of wound healing, it may have a negative effect on wound healing because it increases inflammatory cell infiltration in the advanced process. It was concluded that silver nanoparticles may contribute to wound healing by suppressing inflammation in the later stages of wound healing without affecting angiogenesis.

Table 1. Data of wound diameters after closure of wounds according to groups in macroscopic examination (according to the πr^2 formula).

Animal / Groups	Crystalline 4. Day	Control 4.Day	Colloidal Silver 4. Day	Crystalline 8. Day	Control 8.Day	Colloidal Silver 8. Day	Crystalline 12. Day	Control 12.Day	Colloidal Silver 12. Day
Rabbit-1	78	113	28	50	96	13	3	28	20
Rabbit-2	50	113	28	13	78	13	13	64	3
Rabbit-3	78	154	50	50	96	13	20	50	50
Rabbit-4	113	113	28	50	78	28	3	13	3
Rabbit-5	78	50	38	28	28	28	7	38	13
Rabbit-6	113	78	28	64	113	28	0	3	0
Rabbit-7	50	113	50	7	13	7	0	28	3
Rabbit-8	78	113	28	13	28	13	1	28	20
Rabbit-9	50	113	50	20	64	13	1	38	1
Rabbit-10	78	78	38	38	28	20	7	50	7
Rabbit-11	50	78	50	28	50	20	0	28	1
Rabbit-12	50	78	28	20	38	13	3	28	3
Rabbit-13	50	113	50	28	78	28	1	28	0
Rabbit-14	50	78	38	20	28	1	3	20	1
Rabbit-15	50	78	50	28	64	20	3	20	7
Rabbit-16	78	113	28	13	50	7	3	38	3
Rabbit-17	50	78	50	38	78	13	1	20	1
Rabbit-18	78	64	50	38	64	20	7	13	3
Rabbit-19	28	28	20	28	50	28	1	20	1
Rabbit-20	50	133	78	50	96	20	0	3	1
Rabbit-21	50	28	20	28	50	3	3	13	0

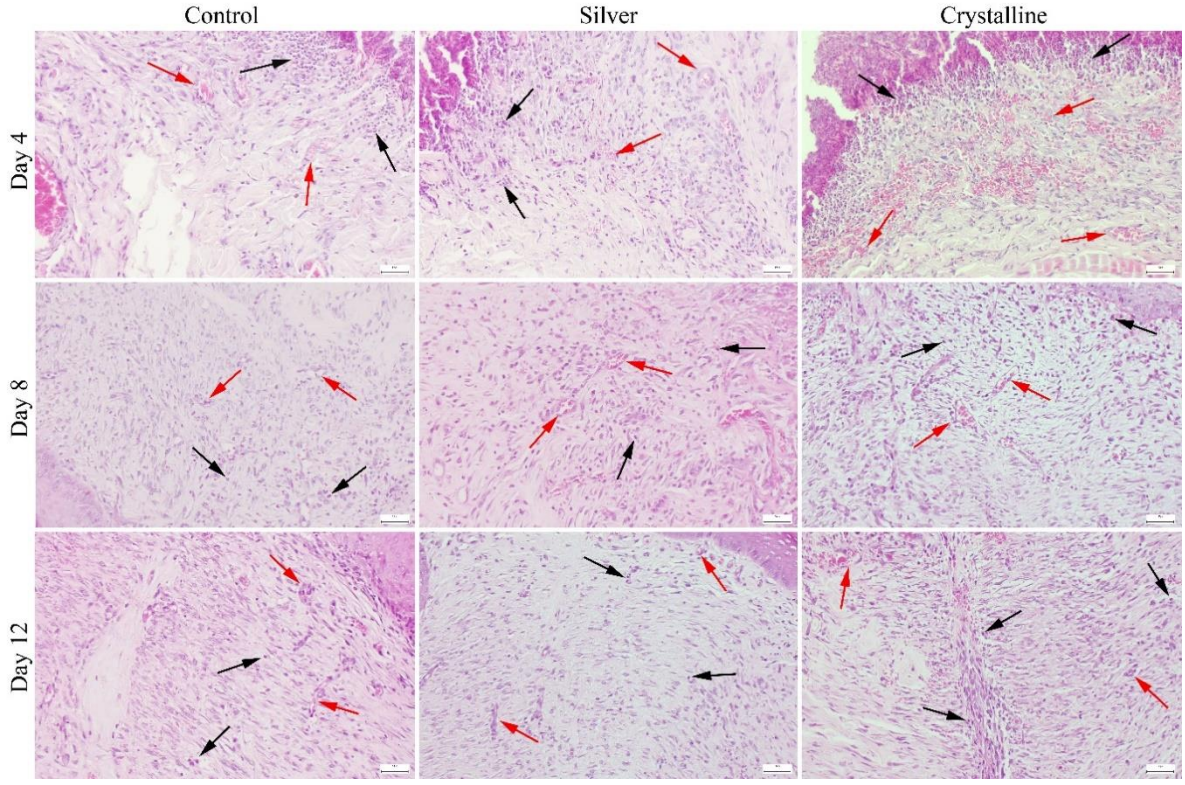

Figure 1: Appearance of angiogenesis (red arrows) and inflammatory infiltrations (black arrows) in the wound area on days 4, 8, and 12 in control, silver, and crystalline groups. H&E staining. Bars: 50 μm.

Table 2. Statistical data between groups for histopathological parameters.

Day	Group	Angiogenesis	Inflammation
4	**Control**	13.50 ± 1.84[a]	3.333 ± 0.422[a]
	Silver	12.17 ± 1.99[a]	4.000 ± 0.000[a]
	Crystallin	23.17 ± 1.99[b]	3.833 ± 0.167[a]
	P value	**0.002**	0.206
8	**Control**	20.83 ± 1.35[a]	2.833 ± 0.543[a]
	Silver	29.67 ± 1.78[a]	3.167 ± 0.543[a]
	Crystallin	27.33 ± 5.06[a]	2.833 ± 0.543[a]
	P value	0.163	0.883
12	**Control**	19.33 ± 2.39[a]	1.833 ± 0.307 [ab]
	Silver	27.00 ± 2.99[a]	1.000 ± 0.000 [a]
	Crystallin	22.00 ± 2.88[a]	2.333 ± 0.422 [b]
	P value	0.172	**0.022**

a,b : The statistical difference between the means with different letters in the same row is significant.

DISCUSSION

Wound products are of great importance in wound-related research and applications. Wound care products contribute to the natural healing process by protecting the tissue and activating cell production (Valenta et al, 2004). In addition to wound dressings and creams, silver is used in medical devices and in the coating of antibiotics (Jean-Yves et al., 2013). Until the invention of antibiotics, silver was used both for food preservation and as a good therapeutic agent. Today, it is known that silver has antibacterial effect on nearly six hundred bacteria (Fung et al., 1996). Nanoparticle silver is obtained by separating silver metal into very small pieces in the order of nanometre. In this way, silver nanoparticles can be

transported to very remote points in the living body through fluids. Silver has antibacterial effect as well as antiallergic effect. Ionic silver is formed by introducing silver into a liquid in the form of Ag + ions. This liquid is called 'Colloidal Silver' in the sense of suspended. Since colloidal ionic silver atoms are positively charged, they are suspended in the liquid by pushing each other. This liquid can be applied to the skin or administered sublingually, by drinking or through the rectum to ensure absorption. Dose and concentration studies for intravenous applications are ongoing (Rosenblatt et al., 2009; Deery, 2009; Lancaster and Steady, 2010; Wesley, 2009). Silver and most silver compounds have oligodynamic effect (Lansdown, 2006). The effectiveness of silver compounds as antiseptics is that the biologically active silver ion (Ag+) irreversibly damages key enzyme systems in the cell membranes of pathogens (Lansdown, 2006). Our study's focus on the healing efficacies of colloidal silver and crystalline silver treatments aligns with the rich history of silver's medicinal use, offering insights into its role within modern wound care paradigms.

In a seminal study by Pansara et al. (2020), the efficacy of a chitosan-based film, enriched with chitosan-stabilized silver nanoparticles (CH-AgNP-CHF), on promoting wound healing was meticulously assessed. This experimental investigation was conducted on 24 male Wistar rats, each inflicted with a standardized wound of dimensions 1.5 x 1.5 cm, subsequently infected with the Escherichia coli strain to simulate a bacterial infection scenario. The subjects were systematically divided into four distinct groups, comprising six rats each, to evaluate the therapeutic potential of various treatments. These included a control group treated with sterile gauze, a group receiving a chitosan solution devoid of nanoparticles (empty chitosan film) as a placebo, a group treated with the CH-AgNP-CHF4 film, and a final group administered with MSN-G, a commercial gel containing 0.002% nanocrystalline silver. The comparative analysis of wound healing efficacy from the initial day to the 21st day post-treatment revealed a hierarchical order of effectiveness, prominently showcasing the CH-AgNP-CHF4 film as superior, followed by MSN-G, the empty chitosan film, and the sterile gauze. Noteworthy observations were made by the 14th day, where the CH-AgNP-CHF4 film and MSN-G treatments had almost entirely facilitated wound healing, mirroring the findings from our concurrent study that indicated a rapid and near-complete healing by day 12 in wounds treated with colloidal silver.

Further detailed observations by Pansara et al. (2020) included wound closure rates (WCR) at various intervals. On the third day, CH-AgNP-CHF4 film demonstrated significantly higher WCR at 65.30%, in stark contrast to the lower percentages noted in other groups, which progressively increased by the 5th and 7th days, reaching near-complete closure by day 14, and achieving full closure by day 21 in the CH-AgNP-CHF4 film and MSN-G groups. These findings were corroborated by histopathological analyses, indicating keratinization in the CH-AgNP-CHF4 and MSN-G treated groups, suggestive of accelerated wound healing facilitated by silver's presence. Our study's outcomes align closely with those of Pansara et al., albeit with an emphasis on colloidal silver's pronounced effect on expedited wound healing observed as early as the fourth day. However, it was noted that while crystalline silver applications initially promoted angiogenesis, contributing positively to the early stages of wound healing, there could be potential adverse effects related to inflammatory cell infiltration in later stages. Nonetheless, it was concluded that silver nanoparticles might effectively enhance wound healing by modulating inflammation during the critical latter stages, without detrimentally impacting angiogenesis.

Masood et al. (2019) evaluated the effects of nanoparticle silver impregnated chitosan-PEG (polyethylene glycol) hydrogel application on wound healing in wounds created in diabetic rabbits. A total of two wounds of 20 mm in size were created on both sides of the midline on the back of the rabbits. In this way, 5 different groups were formed. In the 1st group (negative control), no treatment was applied to the rabbits. In the second group (positive control), the wounds were treated with nitrofurazone (0.2% w/w). Groups 3, 4 and 5 were treated with AgNP (Nanoparticle silver), chitosan - PEG hydrogel only and chitosan-PEG hydrogel containing AgNP, respectively. Wound sites were closed with a standard surgical dressing and re-dressed on days 4, 8 and 12. According to the results of the study, according to both macroscopic and histopathological findings, it was reported that wound healing was faster and more effective in the silver-containing groups compared to the negative control group. In our study, as in this study, colloidal silver was found to be faster and more effective in wound healing compared to the control group.

Hypochlorous acid (HOCl) is a non-cytotoxic microbiocidal agent active against all bacterial, viral, and fungal human pathogens (Mekkawy and Kamal, 2014). HOCl is formed by combining acidic chlorine oxide (Cl) with water. Stabilised in the form of a physiologically stable solution, HOCl is a naturally occurring molecule produced by neutrophils to destroy pathogens (Gold et al., 2019). It has an excellent bactericidal effect against various microorganisms due to its high oxidising capacity (Tsai et al., 2023). Kuwabara et al. (2018) created chronic wounds infected with Pseudomonas aeruginosa in diabetic mice and used HOCl solution for 12 days to prevent infection and wound treatment in these chronic wounds. In this study, a 1 full thickness wound was created on the back with a sterile 8 mm dermal punch biopsy tool and bacterial

inoculation with Pseudomonas aeruginosa strain at a density of 1.0 × 106 CFU / ml was performed after the wound was created. As a result of this study, it was reported that cleaning the wounds with HOCl solution resulted in a slight delay in wound repair compared to the control group, no significant difference in histopathological examinations and a significant decrease in P. aeruginosa bioburden. Our study contrasted with this study, and it was found that HOCl solution accelerated wound healing compared to the control group.

Kuwabara et al. (2020) explored wound healing in diabetic mice with Pseudomonas aeruginosa infections, focusing on treatments with hypochlorous acid (HOCl) and a novel coating of nanoparticle silver (Ag NPs) with chitin-nanofibre sheets (CNFS). The research meticulously prepared the HOCl solution and nanoparticle silver, ensuring precise concentration and formulation for optimal wound healing assessment. Through creating standardized full-thickness wounds and treating these with varying combinations of HOCl, Ag NPs, and CNFS, the study meticulously evaluated the therapeutic efficacy over a 12-day period, comparing outcomes against a non-cleaned control group. The methodology involved daily treatment regimens and consistent monitoring of wound closure rates, providing a comprehensive dataset on the healing progression. Notably, the study found that all treated groups, except the non-cleaned control, showed significantly enhanced wound healing, with the combination of HOCl and CNFS/Ag NP standing out for its pronounced beneficial impact. This group exhibited superior healing, supported by both macroscopic assessments and histological examinations highlighting reduced granulation tissue formation—a key indicator of effective wound management. Our investigation aligns with Kuwabara et al.'s findings, particularly noting the accelerated wound healing facilitated by HOCl solution across multiple observation points. This congruence underscores the potential of HOCl, both alone and in conjunction with nanoparticle silver and CNFS, in promoting wound healing in challenging diabetic models infected with Pseudomonas aeruginosa. These results not only validate the therapeutic promise of these compounds but also contribute to the broader understanding of their mechanisms and applications in advanced wound care strategies.

CONCLUSION

In conclusion, our findings offer compelling evidence for the use of colloidal silver and hypochlorous acid as effective agents in the management of dermal wounds. By accelerating wound closure and enhancing the quality of wound repair, these agents hold significant promise for improving patient outcomes in wound care. Future research should focus on optimizing formulations and delivery methods to maximize therapeutic benefits, alongside exploring their effects in diverse wound types and clinical settings. Our study contributes to the growing body of literature advocating for the incorporation of these agents into wound management protocols, potentially setting new benchmarks in the field of regenerative medicine and wound care.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: HÇ contributed to the project idea. HÇ, AGK and MDT contributed design and execution of the study. HÇ and İTD contributed to the acquisition of data. HÇ and Vİ analysed the data. HÇ, Vİ and İTD drafted and wrote the manuscript. BS, ED and MK reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Burdur Mehmet Akif Ersoy University Reserch Animals Application Center. This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University (MAKU-HADYEK, Ref No: 73/613, Tarih: 15/01/2020).

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RESEARCH ARTICLE

Quality Features of Afyon Fermented Sausage (Sucuk) and Standards Compliance

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ABSTRACT

Sucuk is one the indispensable tastes of Turkish cuisine that is preferred by a wide range of consumers. Hence, it must be produced in accordance with standards. In this study, it is aimed to specify the physicochemical and microbiological characteristics of Afyon Sucuk and their suitability to Communiqué on Turkish Food Codex Meat and Meat Products, Turkish Food Codex Microbiological Criteria Regulation and TS 1070 Sucuk Standard. As a result of our research, it is detected that in terms of pH, protein and fat contents; 83.3%, 26.66% and 16.66% of samples are not in the limits that are specified in the Turkish Food Codex Notification of Meat and Meat Products. On the other hand, the ash and salt contents of samples are determined to be in the limits of Communiqué on Turkish Food Codex Meat and Meat Products. In terms of total aerobic mesophilic bacteria and total yeast/ mold counts, 60% of samples had higher values than the specified limits in TS 1070. Total coliform counts, *Staphylococcus aureus, Escherichia coli, Salmonella spp.* and *Listeria monocytogenes* counts were found to be in accordance with Turkish Food Codex Microbiological Criteria Regulation and TS 1070 (TSE Turkish Fermented Sausage Standard).

Keywords: Afyonkarahisar, fermented sausage, microbiology, pH, quality

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Afyon Sucuğunun Kalite Özellikleri ve Standartlara Uygunluğu

ÖZ

Sucuk, Türk mutfağının vazgeçilmez lezzetlerinden biridir ve geniş bir tüketici kitlesi tarafından tercih edilmektedir. Bu nedenle, sucuğun kalite standartlarına uygun olarak üretilmesi önemlidir. Bu çalışmada Türkiye'de önemli bir yere ve pazara sahip olan Afyon sucuğunun fizikokimyasal ve mikrobiyolojik kalite kriterlerinin belirlenerek, Türk Gıda Kodeksi Et ve Et Ürünleri Tebliği, Türk Gıda Kodeksi Mikrobiyolojik Kriterler Yönetmeliği ve TS 1070 Sucuk Standardına uygunluğunun araştırılması amaçlanmıştır. Araştırma sonucunda örneklerimizin pH değeri bakımından, %83.3'ünün, protein değeri bakımından %26.66'sının, yağ değeri bakımından %16.66'sının Türk Gıda Kodeksi Et ve Et Ürünleri Tebliğinde belirtilen sınırlar dahilinde olmadığı belirlenmiştir. Ek olarak tüm örneklerimizin kül ve tuz miktarları bakımından Türk Gıda Kodeksi Et ve Et Ürünleri Tebliğinde belirtilen sınırlar içerisinde tespit edilmiştir. Ayrıca örneklerimizin toplam aerobik mezofilik bakteri ve toplam maya/küf sayıları bakımından %60'ının, TS 1070 sucuk standardında belirtilen sınırlardan daha yüksek olduğu, Toplam koliform grup bakteri sayısı, *Staphylococcus aureus, Escherichia coli, Salmonella spp.* ve *Listeria monocytogenes* türü bakteri sayılarının ise Türk Gıda Kodeksi Mikrobiyolojik Kriterler Yönetmeliği ve TS 1070 Sucuk Standardına uygun olduğu ortaya konulmuştur.

Anahtar kelimeler: Afyonkarahisar, fermente sosis, mikrobiyoloji, kalite, pH

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INTRODUCTION

Sucuk is a popular semi-dry fermented meat product that is mainly produced with the mixture of beef, buffalo, beef fat, sheep lard, salt, nitrate/ nitrite and various seasonings (Gökalp et al. 1997). Sucuk is a traditional delicious taste of Turkish cuisine that is preferred by a wide range of consumers. Production of sucuk must be in accordance with standards with regards to public health and product quality. Therefore, the investigation of the manufacturing process of sucuk and evaluation of quality features are crucial (Kılıç 2009).

In spite of the manufacturing nearly in every region of Turkiye, Afyon and Kayseri are the prominent cities in sucuk production (Artam 2018). Camel meat, buffalo meat, sheep and goat meats are also used in sucuk production in Turkiye other than beef. Rather than primary cuts, low-value cuts are commonly preferred in sucuk production (Sönmez 1986).

The lineament of Afyon sucuk is the use of significant proportions of buffalo meat in addition to beef in sucuk formulation. Afyon sucuk contains less spice and its taste is more pronounced. The unique flavour of Afyon sucuk results from the meat of buffalos that are grown in that region. Mentioned buffalos fed with aromatic plants as well as poppy seed meal. Hence, Afyon sucuk does not dry out easily while cooking and its ingestion is more efficient. As a consequence of its own undisturbing odor, it does not cause any indigestion (Artam 2018).

Afyonkarahisar region is one of the most important sucuk production centers in Turkiye and sucuks that have manufactured in this region have a large market share. Nevertheless, there are not many research about sucuk production in Afyonkarahisar region and its adherence to quality standards.

In this study, it is aimed to investigate the physicochemical and microbiological quality criteria and their compliance with Communiqué on Turkish Food Codex Meat and Meat Products, Turkish Food Codex Microbiological Criteria Regulation and TS 1070 Sucuk Standard.

MATERIAL and METHOD

Material

30 sucuk samples purchased from 30 different sucuk processing plants that have operating permits taken from the Ministry of Agriculture and Forestry according to Communiqué on Turkish Food Codex Meat and Meat Products (Communiqué No. 2012/74 and Communiqué No. 2015/7) within the scope of this study.

This study was carried out in two recidivisms, considering the two different production dates of the samples that were purchased from sales points. These samples were transported to the laboratory in sealed polyethylene bags within a cold chain and then stored at refrigeration temperatures until the analysis was completed.

Methods

Physicochemical analysis

pH value is one of the most important parameters that directly influences water holding capacity and shelf life of fermented meat products (Çiçek and Polat 2016). pH measurements were performed by a pHmeter (Ohaus, starter 3100) that was previously calibrated with pH 4.0 and 7.0 solutions (Gökalp et al 2001).

Dry matter contents of samples were determined according to AOAC 990.20 (AOAC 2016).

Samples were incinerated in a muffle furnace (Elektromag M 1811) at about 550 °C for 6-8 h and then cooled in a desiccator and weighed (AOAC 2016).

Fat contents were determined with Soxhelet apparatus using hexane as solvent (Ertaş et al. 2019).

Kjeldahl method was used to determine protein contents. Calculated nitrogen contents were multiplied by 6,25 to obtain protein contents (%) (Özyurt 2018).

Water activity values were determined by using Novasina LabTouch-aw (Lachen, Switzerland) (AOAC 2016).

Salt Content analysis

The salt contents of samples were determined according to the Mohr titration. 5 gr of the sample was solved with 50 ml distilled water. 0,5 ml of 5 % solution of potassium chromate indicator was added to each mixture and then titrated with standardized silver nitrate solution until a red-brown color persisted for 30 sec (Yalçın and Şeker 2016).

Color Measurements

Color measurements were performed by using a Konica Minolta Chroma Meter CR-400 (Japan) according to Hunter-Lab Color System. Mean values obtained from 5 measurements from inner, outer and lateral surfaces of sucuk samples in terms of lightness (L*), redness (a*) and yellowness (b*) according to the standard conditions of the Commission International d'Eclairage (CIE).

Also;

Hue angle: Ho = tan -1(b*/a*)

Saturation Index (Chroma): C* = (a*)2+(b*)2)1/2

Browning Index: BI = ((100* (x-0.31)) /0.17) values were calculated using L*, a* and b* (Kurtuldu and Özcan 2018).

Texture analysis

Texture Profile Analysis

TPA values were measured by TA.HD Plus Texture Analyser (Stable Micro Systems, Surrey, YL, England) at room temperature using 5 cm3 meat cubes. The

measurements were done with the following testing procedure: constant speed of 1mm/ s (pre-test), 5 mm/s (test) and 5 mm/s (post-test). A cylindrical 50 mm-diameter probe was used at a 20% compression rate (De Huidobro et al 2005).

Warner-Bratzler Shear Force

Shear force analysis of sucuk samples was performed with TA.HD Plus Texture Analyser (Stable Micro Systems, Surrey, YL, England). Square sections taken from samples were analysed using a WB shear blade probe (Ceylan 2022).

Microbiological Analysis

Microbiological analyses were performed with the spread plate method. 10 g of sample diluted in 90 ml Ringer's solution (Merck, 11525, Germany) and homogenized in a stomacher (Lab Stomacher Blender 400-BA 7021, Seward Medical). Dilution series were prepared by taking 1 mL from this homogenate. All spreading procedures were performed in double parallel runs and results are given in log CFU/g (Sekin and Karagözlü 2004).

The total mesophilic aerobic bacteria number was determined using Plate Count Agar (PCA) (Merck 1.05463, Germany). Inoculated plates were incubated at 30°C for 48-72 h (ISO 2008, ISO 2013). Man Rogasa and Sharpe Agar (MRS) (Merck 1.10661) was used to determine LAB counts, followed by incubation at 30°C for 24-48 h in an anaerobic jar (Merck 1.16387) (Kneifel 1994).

Yeast and mold counts were performed using Rose Bengal Chloramphenicol Agar (Merck 1.00467, Germany) (RBC). Inoculated petri dishes were incubated at 22°C for 5-7 days aerobically (ISO, 2013b). Total coliform counts were determined with Violet Red Bile Agar (Merck 1.01406, Germany) incubating aerobically at 30°C for 24 to 48 h (ISO 2015).

For *Staphylococcus aureus*, Baird Parker Agar (Merck 1.05406, Germany) and for *E. coli*, Chromocult TBX Agar (Merck 1.16122, Germany) were used. The plates were incubated aerobically at 30-35°C and 37°C for 24-48 h, respectively. At the end of incubation period, suspected colonies (black colonies with white margins) were picked and inoculated onto Baird Parker Agar once again with the same incubation conditions.

The growing colonies were subjected to a coagulase test (Bactident Coagulase, Merck 1.13306, Germany) and the positive ones were confirmed as *S. aureus*. For *E.coli* confirmation, the growing colonies were examined under UV light (366 nm) (ISO 1999, ISO 2001).

Salmonella spp. counts were determined by spread plates using Nutrient Broth (NB) (Merck 1.05443, Germany) Rappaport Vassiliadis Salmonella Enrichment Broth (RVS) (Merck 1.07700, Germany) and Brilliant Green Phenol Red Lactose Sucrose Agar (BPLS) (Merck 1.10747, Germany) and Xylose Lysine Deoxycholate Agar (XLD) (Merck 1.105287, Germany). Inoculated petri dishes were incubated aerobically at 37°C for 24-48 h (Greenwood et al. 1984, Flowers et al. 1992, ISO 2017).

Listeria monocytogenes counts were determined by plating on Fraser broth (Merck 1.10398, Germany) and Oxford Agar (Merck 1.07004, Germany) then incubation at 37°C for 24-48 h (ISO 2017).

Statistical Analysis

The study's results were achieved by performing two replicates and all analyses were done in two repetitions. The SPSS program version V 23.0.0 was used for the variance analysis. The significant levels of difference were determined using Duncan's multiple-range tests ($P<0.05$).

RESULTS

Physicochemical Analyses

pH values of sucuk samples are shown in Table 1. pH values ranged from 4.61 to 6.82 ($P<0.05$). The mean pH value is determined to be 6.21.

Table 1 shows the mean dry matter contents of 30 sucuk samples purchased from the sales points of the sucuk production plants with operating permits taken from the Ministry of Agriculture and Forestry. Dry matter values range between 41.83% and 69.11% ($P<0.05$). The mean dry matter content is determined to be 55.013%.

Ash contents of samples vary from 2.53 to 4.11%. The mean ash value is determined to be 3.22 %. ($P<0.05$; Table 1). About 13 samples (43.33%) are involved in the highest ash content range that varied from 3.0 to 3.5%. Only one sample (3.33%) was detected to be in the lowest ash content range that is in the >4.5%. None of the samples are detected to be in the <2.5%range.

Water activity values of samples vary between the range of 0.458-0.907 with a mean value of 0.785 ($P<0.05$; Table 1). About 13 samples (43.33 %) are involved in the highest ash content range that vary from 3.0 to 3.5%. Only one sample (3.33%) was detected to be in the lowest ash content range that is in the >4.5%. None of the samples were detected to be in the <2.5% range. According to Table 1, the highest aw value range consists of 12 samples (40%) between 0.800 to 0.900 whereas the lowest aw value with only 1 sample (3.33%) stands in the <0.500 range.

Minimum, maximum and mean fat contents of sucuk samples are determined to be 10.87%, 44.01% and 29.37%, respectively ($P<0.05$; Table 2).

The minimum, maximum and mean protein contents of 30 samples are as follows; 8.31%, 19.42% and 14.99%, respectively ($P<0.05$; Table 2).

Table 1. Number of sucuk samples and percentages in terms of physicochemical analysis and, pH, dry matter and ash contents

pH	Sample		Dry Matter (%)	Sample		Ash Content (%)	Sample		a_w	Sample	
	Number	%		Number	%		Number	%		Number	%
>6.0	23	76.66	>60	2	6.66	>4.0	1	3.33	>0.900	5	16.66
5.4-6.0	2	6.66	55-60	14	46.66	3.8-4.0	2	6.66	0.800-0.900	12	40
5.0-5.4	3	10	50-55	9	30	3.5-3.8	5	16.66	0.700-0.800	8	26.66
4.7-5.0	2	6.66	45-50	4	13.33	3.0-3.5	13	43.33	0.600-0.700	4	13.36
4.5-4.7	-	-	40-45	1	3.33	2.5-3.0	9	30	0.500-0.600	-	-
<4.5	-	-	<40	-	-	<2.5	-	-	<0.500	1	3.33
Minimum	4.61		Minimum	41.83		Minimum	2.53		Minimum	0.458	0.458
Maximum	6.82		Maximum	69.11		Maximum	4.411		Maximum	0.907	0.907
Mean	6.21±0.53		Mean	55.013±5.13		Mean	3.22±0.39		Mean	0.785 ±0.1 10	0.785± 0.110

Table 2. Number of sucuk samples and percentages in terms of physicochemical analysis and fat, protein and salt contents (n=30).

Fat content (%)	Sample		Protein (%)	Sample		Salt Content (%)	Sample	
	Number	%		Number	%		Number	%
>40	3	10	>19	2	6.66	>3,5	1	3.33
35-40	4	13.36	18-19	2	6.66	3.0-3.5	4	13.36
30-35	4	13.36	17-18	-	-	2.5-3.0	11	36.66
25-30	12	40	16-17	3	10	2.0-2.5	14	46.66
20-25	5	16.66	15-16	9	30	1.5-2.0	-	-
<20	2	6.66	<15	14	46.66	<1.5	-	-
Minimum	10.87		Minimum	41.83		Minimum	2.22	
Maximum	44.01		Maximum	69.11		Maximum	3.55	
Mean	29.37±7.20		Mean	55.013±5.13		Mean	2.64±0.32	

Salt Content

Salt contents of sucuk samples are shown in Table 2. Minimum, maximum and mean salt contents of the samples are detected to be as 2.2%, 3.55% and 2.64%, respectively.

Color Values

L* values of 30 samples are shown in Table 3. Maximum, minimum and mean L* values are; 55.83, 42,85 and 48.98, respectively ($P<0.05$). Hue angle values of sucuk samples are ranged between 25.77 and 53.14 ($P<0.05$) while chroma values are determined to be between 25.47 and 37.43 ($P<0.05$) as shown in Table 4. Minimum, maximum and mean whiteness index values of samples are determined to be 725.75, 1182.33 and 937.50, respectively ($P<0.05$).

Table 3. Number of sucuk samples and percentages in terms of physicochemical analysis and Color values (n=30).

L* Values	Sample		a* Values	Sample		b* Values	Sample	
	Number	%		Number	%		Number	**%**
>55	1	3.33	>30	1	3.33	>25	3	10
50-55	8	26.66	25-30	20	66.66	20-25	9	30
45-50	17	56.66	20-25	8	26.66	15-20	16	53.33
40-45	4	13.36	15-20	1	3.33	10-15	2	6.66
35-40	-	-	10-15	-	-	5-10	-	-
<35	-	-	<10	-	-	<5	-	-
Minimum	42.85		Minimum	15.28		Minimum	13.22	
Maximum	55.83		Maximum	30.32		Maximum	25.89	
Mean	48.98±3.09		Mean	25.49±2.76		Mean	19.07±3.14	

Table 4. Number of sucuk samples and percentages in terms of physicochemical analysis and Color values (n=30) (Continued).

Hue Angle	Sample		Chroma values	Sample		WI	Sample	
	Number	%		Number	%		Number	%
>45	2	6.66	>34	5	16.66	>1100	2	6.66
45-40	8	26.66	34-33	6	20	1050-1100	3	10
35-40	8	26.66	32-33	2	6.66	1000-1050	4	13.36
30-35	9	30	31-32	6	20	950-1000	5	16.66
25-30	3	10	30-31	7	23.33	900-950	10	3.33
<25	-	-	<30	4	13.36	<900	6	20
Minimum	25.77		Minimum	25.47		Minimum	725.75	
Maximum	53.14		Maximum	37.43		Maximum	1182.33	
Mean	36,80±5.93		Mean	31.99±2.65		Mean	937.50±2.65	

Textural analysis

Texture Profile Analysis and Warner-Bratzler Shear Force

The mean textural properties of 30 sucuk samples are as follows; hardness; 5933.86 N, adhesiveness;-88.31, springiness; 0.727 mm, cohesiveness; 0.575, gumminess; 3440.723N, chewiness; 2495.546 N and resilience; 0.18 (Table 5).

Maximum, minimum and mean Warner-Bratzler shear forces are determined to be 8201.02 kgf, 88.43 kgf and 145.30 kgf, respectively.

Microbiological Analysis

Total Aerobic Mesophilic Bacteria Counts

Total Aerobic Mesophilic Bacteria Counts of sucuk samples are determined to be as; minimum; <1 log cfu/g, maximum; 2.93 log cfu/g and mean 1.90 cfu/g ($P<0.05$; Table 6).

Lactic Acid Bacteria (LAB) Counts

LAB counts of 30 sucuk samples are determined to be in the range of 1.85 to 7.63 log cfu/g, with a mean of 6.21 log cfu/g ($P<0.05$; Table 6).

Total Yeast and Mold Counts (TYM)

Total mold and Yeast counts are determined to be in the 0.70- 4.65 log cfu/g range, with an average of 2.46 log cfu/g ($P<0.05$; Table 6).

Total Coliform Bacteria Counts

Total Coliform Count (TCC) of samples were ranged between <1 log cfu/g and 1.95 log cfu/g ($P<0.05$; Table 6).

***Staphylococcus aureus* counts**

S. aureus counts of sucuk samples were ranged between <1.0 log cfu/g- 2.07 log cfu/g with a mean count of 0.53 log cfu/g ($P<0.05$; Table 6).

Table 5. Number of sucuk samples and percentages in terms of textural properties and texture analysis results (n=30).

Hardness (N)	Sample		Adhesiveness	Sample		Springiness (mm)	Sample		Cohesiveness	Sample	
	Number	%		Number	%		Number	%		Number	%
>8500	2	6.66	>-50	4	13.36	>900	1	3.33	>0.700	3	10
7500-8500	6	20	-100 - -50	17	56.66	900-800	9	30	0.700-0.600	10	33.33
6500-7500	4	13.36	-150 - -100	4	13.36	800-700	10	33.33	0.600-0.500	10	33.33
5500-6500	5	16.66	-200 - -150	3	10	700-600	7	23.33	0.500-0.400	6	20
4500-5500	5	16.66	-250 - -200	2	6.66	600-500	3	10	0.400-0.300	1	3.33
<4500	8	26.66	<-250	-		<500			<0.300	-	-
Minimum	3609.12		Minimum	-205.47		Minimum	0.566		Minimum	0.365	
Maximum	8633.41		Maximum	-33.25		Maximum	0.909		Maximum	0.715	
Mean	5933.86±1657.84		Mean	-88.31±47.46		Mean	0.727±0.10		Mean	0.575±0.09	
Gumminess (N)	Sample		Chewiness (N)	Sample		Resilience	Sample		Warner-Bratzler Shear Force	Sample	
	Number	%		Number	%		Number	%		Number	%
>6000	2	6.66	>4000	2	6.66	>0.25	4	13.36	>180	5	16.66
5000-6000	2	6.66	3500-4000	3	10	0.20-0.25	7	23.33	160-180	6	20
4000-5000	5	16.66	3000-3500	3	10	0.15-0.20	8	26.66	140-160	7	23.33
3000-4000	8	26.66	2500-3000	4	13.66	0.10-0.15	9	30	120-140	3	10
2000-3000	10	33.33	2000-2500	8	26.66	0.10-0.05	2	6.66	100-120	6	20
<2000	2	6.66	<2500	10	33.33	<0.05	-	-	<100	3	10
Minimum	1731.356		Minimum	1238.396		Minimum	0.09		Minimum	88.43	
Maximum	6143.28		Maximum	5313.97		Maximum	0.28		Maximum	204.02	
Mean	3440.723±1221.267		Mean	2495.546±954.97		Mean	0.18±0.05		Mean	145.30±32.99	

Table 6. Number of sucuk samples and percentages in terms of microbiological properties and microbiological counts (n=30) (log cfu/g).

TAMB Count	Sample		LAB Count	Sample		Coliform Count	Sample		TYM Count	Sample		*S.aureus* Count	Sample	
	Number	%		Number	%		Number	%		Number	%		Number	%
>2.4	7	23.33	>7	16	53.3	>2	-	-	>3.0	9	30	>2	2	6.66
2.4-2.0	13	43.33	7.0-6.0	8	26.6	1.8-2.0	2	6.66	2.5-3.0	3	10	1.6-2.0	-	-
2.0-1.6	6	20	6.0-5.0	3	10	1.6-1.8	-	-	2.0-2.5	6	20	1.4-1.6	3	10
1.6-1.3	-	-	5.0-4.0	1	0.33	1.4-1.6	2	6.66	1.5-2.0	5	16.66	1.2-1.4	3	10
1.3-1	-	-	4.0-3.0	1	0.33	1.0-1.4	3	10	1.0-1.5	3	10	1.0-1.2	4	13.66
<1	4	13.66	<3.0	1	0.33	<1.0	23	76.6	<1.0	4	13.66	<1.0	18	60
Minimum	<1		Minimum	1.85		Minimum	<1.0		Minimum	0.70		Minimum	<1.0	
Maximum	2.93		Maximum	7.63		Maximum	1.95		Maximum	4.65		Maximum	2.07	
Mean	1.90±0.90		Mean	6.21±1.81		Mean	0.32±0.67		Mean	2.46±1.00		Mean	0.53±0.73	

DISCUSSIONS

Physicochemical Analyses

The ripening period of sucuk production consists of two main stages; fermentation and drying. During fermentation, Lactic acid bacteria metabolize glucose, the main energy source, to lactic acid. As a result, the pH declines, changing the texture and flavour (Bover-Cid 2001). Besides, water loss increases and the product dries out in a short time. Meanwhile, nitrite reduction occurs and desirable color and aroma formation accelerate and also the microbial deteriorations are inhibited (Gökalp et al. 1997). According to Communiqué on Turkish Food Codex Meat and Meat Products (Communiqué No. 2012/74) the maximum pH value of fermented sucuk should be 5,4 and it should be 5,6 for heat treated sucuk (Anonymous 2012). Consequently, 25 of the samples (about 83.3%) are above the stated limit in Communiqué on Turkish Food Codex Meat and Meat Products.

Following slaughter, the pH value of meat ranges between 7,1- 7,3; about 6 or 8 h later pH decreases to 5,6-5,7 as a result of rigor mortis. The high pH values of some sucuk samples are probably because of using meat that has not completed its Rigor mortis stage yet or DFD Meat (Dark Firm Dry) that has high water holding capacities. The use of excessive additives, particularly acid regulators, could be another factor.

Pehlivanoğlu et al. (2015) reported that the pH values of 12 of 30 sucuk samples (40%) that were supplied from İstanbul region were out of legal limits. They determined the minimum pH value as 4.21; the maximum pH value as 5.71 and the mean pH value was found to be 5.21. Similarly, Öksüztepe et al. determined the minimum, maximum and mean pH values of sucuk samples from Elazığ region were found to be 4.75, 6.76 and 5.18, respectively.

Current results are different from these previous studies. These differences may be on account of the different raw materials, differences on the type and amount of additives and also differences on production process, process conditions etc. Dry matter content in sucuk is one of the most important quality parameters that also affects consumer admirations.

According to the limit that set by Communiqué on Turkish Food Codex Meat and Meat Products, the ratio of moisture content to total meat protein must be less than 2,5; this ratio must be less than 3,6 in heat treated sucuk. One of 30 sucuk samples (3,33%) has the ratio above 3.6, whereas 27 of 30 samples (90%) have the ratio above 2.5. The high moisture content of samples may be a result of a short heat treatment followed by an insufficient drying process. These results are similar to those of others who also found dry matter contents in the range of 48.52% - 64.37% with a mean dry matter content of 56.72% (Özfiliz et al. 2018) and another study in the range of 33.09% - 74.03% with a mean value of 61.25% (Öksüztepe et al. 2011).

Weight loss due to moisture loss is one of the most significant losses in terms of product cost. Producers often use food additives with moisture retention properties and/or they marketize the products just before the whole production period. Our results are in the same line with these assumptions.

The formulation of sucuk, types and ratios of additives directly affect the ash contents of products. Additionally, the ultimate ash amounts are influenced significantly by the species, age and fattening type of animal, besides the muscles used in manufacture. Communiqué on Turkish Food Codex Meat and Meat Products does not contain any statements about ash limits. On the other hand, TS 1070 Sucuk Standard indicates that ash contents must be 2-5% for fermented sucuk. Ash contents of the current study conform with this regulation. Despite complying with the TS 1070 Sucuk Standard, these results are lower than previous findings by Erdoğrul and Ergün (2005), Sancak et al. (1996) and Öksüztepe et al. (2011) that indicated mean ash contents of sucuk samples as 5.20%, 3.99% and 5.39%, respectively.

Water activity has nearly the same value as pH in terms of a food's production and evaluation phases. This term expresses the amount of free water which is used for the metabolism and the proliferation of microorganisms in that food. Hence, it is supposed to be a stability indicator in terms of microbiological quality and it plays a significant role in food technology (Yıldırım 1996). Kara et al. (2021) reported aw values varying between 0.954-0.354 for sucuk samples. Differences with these similar studies may be on account of storage conditions and also properties of packaging materials.

The addition of fat is essential for sucuk production due to its ability to enhance the product's flavor and aroma as well as the desired marbling formation. Types, amount and storage conditions of these fats are critical factors from the standpoint of final consumption quality and also consumer acceptance. According to Communiqué on Turkish Food Codex Meat and Meat Products (Communiqué No. 2012/74) the ratio of fat content to total meat protein must be lower than 2,5 for both fermented and heat treated sucuk (Anonymous 2012). 5 samples (16.66%) fat contents were determined to be over this limit and the remaining samples were determined to be within the stated limit. According to TS 1070 Sucuk Standard, the maximum fat contents of I. Grade sucuk must be 35%; II. and III. Grades must be 40% (TS 2002). Therefore, 22 samples (73.33%) fat contents are lower than 35% and 7 (25.33%) samples fat contents are lower than 40%. One (3.33%)

remaining sample's fat content is determined to be more than 40 %. Consequently, 22 samples are confirmed to be I. Grade, 7 samples are confirmed to be II. and III. Grades. The remaining sample is out of this classification. Erdoğrul and Ergün (2005) reported the mean fat content of sucuk as 39,20%, Kolsarıcı et al. (1996) 37.15% and lastly Öksüztepe et al (2011) 35.22%. These values are much more than the present study's fat contents. This finding may be attributed to the alteration of limits in Communiqué on Turkish Food Codex Meat and Meat Products that came into effect in 2012. Producers have decreased the fat contents of sucuk formulations from the specified date according to legislation.

In terms of nutritional value as well as sucuk quality, protein is the most significant meat component. Similar previous studies reported the mean protein contents of sucuk samples as 22.48% (Erdoğrul and Ergün 2005) and 21.92% (Öksüztepe et al. 2011). Current results are partly lower than these previous values. These differences may be due to the differences in the breed, species and age of the animal, besides the used carcass cuts in the sucuk production. Another reason may be the updates in the formulations according to the alterations in Communiqué on Turkish Food Codex Meat and Meat Products.

Salt Contents

Salt content has quite important effects on the taste and aroma of sucuk. Besides enhancing taste, salt also improves the consistency properties of the product. Salt also inhibits microbial growth by reducing water activity (Şimşek et al. 2023). No legal maximum limits have been established for salt content by Communiqué on Turkish Food Codex Meat and Meat Products whereas a 5% maximum level has been set in TS 1070 Sucuk Standard (TS 2002). According to this standard, salt contents of all of the samples are within the established limits. Erdoğrul and Ergün (2005) reported the mean salt contents of sucuk samples in Kahramanmaraş province as 3.01 %. Öksüztepe et al (2011) reported minimum, maximum and mean salt contents of sucuk samples sold in Elazığ province as 1.63%, 6.41% and 4.36%, respectively. The added salt contents of the sucuk samples from different brands vary due to the lack of a legal limit in the regulations. Each brand determines its own salt content in the formulations depending on consumer demands.

Color Values

Color is an efficient factor that affects customer purchase tendency of meat products. The formation of color depends on curing agents, pigments and the reactions of other factors. Meat contains myoglobin, the most important one, hemoglobin, cytochrome flavin and some other color agents (Vural and Öztan 1992). Poçan et al. (2015) reported the L* values of fermented sucuks in the range of 44.91-55.20. Kara et al. (2021) determined the maximum, minimum and mean L* values of sucuk samples as 57.81, 31.34 and 47.80, respectively. Maximum, minimum and mean a* values of current samples were determined to be as 30.32, 15.28 and 25.49, respectively ($P<0.05$). Poçan et al. (2015) reported the a* values in the range of 19.40-25.95. In a similar study Kara et al. (2021) reported the maximum, minimum and mean a* values of sucuk samples as 29.02, 17.69 and 23.45, respectively. The maximum, minimum and mean b* values of our current samples were determined to be as 25.89, 13.22 and 19.07, respectively. Poçan et al. (2015) reported the minimum and maximum b* values as 14.68 and 19.30, whereas Kara et al. (2021) determined the maximum, minimum and mean b* values of sucuk samples as 30.63, 18.45 and 23.21, respectively. Our results are in the same line with these previous studies. Differences in these similar studies may be attributed to differences in formulations, type of sucuk and variations in the processes during production and post-production periods.

Textural analysis

Texture Profile Analysis and Warner-Bratzler Shear Force

Textural properties of food products is one the most important parameters for its quality. Stabilizers, emulgators, thickeners and some other ingredients for structure defending effect the texture of food via various active mechanisms (Khan et al. 2018). Warner-Bratzler shear force is defined as the minimum force that is required to cut the food (Bratcher 2004). In a similar study by Kara et al. (2021) textural properties were reported as: hardness; 5320.21, adhesiveness;-24.24, springiness; 0.78, cohesiveness; 0.62, gumminess; 3338.62, chewiness; 2703.93 and resilience; 0.22.

Microbiological Analysis

Total Aerobic Mesophilic Bacteria Counts

There is no legal limits for sucuk and heat treated sucuk in Turkish Food Codex Microbiological Criteria Regulation and TS 1070 Sucuk Standard (TS 2002). However, in a sucuk that is produced under properly hygienic conditions, TAMB counts should be under 6 log cfu/g (İnal 1992).

In a similar study by Pehlivanoğlu et al. (2015) the minimum TAMB counts were reported to be <1 log cfu/g, maximum counts 2.03 log cfu/g and mean counts 1.23 log cfu/g. Öksüztepe et al. (2011) claimed the minimum, maximum and mean TAMB counts that were sold in the Elazığ region as; 7.48 log cfu/g, 9.90 log cfu/g and 8.75 log cfu/g, respectively.

There have been many studies on this subject until today. Current results are in line with some previous studies (Pehlivanoğlu et al. 2015) whereas they are lower than some other studies (Öksüztepe et al. 2011, Çon et al. 2002).

These variations between current results and previous researches could be the consequence of modifications in production technologies and storage conditions. Microbiological activities play an essential role in the formation of typical properties of sucuk in the ripening period. Microbial load comes from the raw material in the sucuk dough (fat, spices and meat) and starter cultures. Increased acidity, pH and water activity as a result of lactic acid bacteria (LAB) activities, cause decreases in the aerobic mesophilic bacteria counts depending on the alteration in the ripening period (Nazlı 1998, Bozkurt and Erkmen 2002).

Lactic Acid Bacteria (LAB) Counts

One of the most important constituents of sucuk microflora is lactic acid bacteria (LAB). In addition to influencing the formation of desired tastes and aromas, LAB also affects the hygienic quality of sucuk by preventing the growth of additional bacteria through the synthesis of numerous antimicrobial metabolic products. As a result of carbohydrate breakdown during fermentation, pH decreases with the accumulation of organic acids, mainly lactic acid. Lactobacillus become the dominant microflora in the media due to the decreased water activity with the addition of salt into the sucuk dough and ripening temperature. Lactobacillus not only decreases the pH, but also effects the taste and aroma (Özdemir 1999, Fadda et al. 1999). Nevertheless, Turkish Food Codex Microbiological Criteria Regulation (TGK 2012) or TS 1070 Sucuk Standard (2002) have no limitations on the LAB counts that should be present in sucuk and heat-treated sucuk.

Previously conducted studies by Çon and Gökalp (1998), Pehlivanoğlu et al. (2015) and Öksüztepe et al. (2011) reported the mean LAB counts as; 8.66 log cfu/g, 7 log cfu/g and 8.56 log cfu/g, respectively. Current results are lower than these reported ones.

High amounts of LAB in sucuk may cause excessive decreases in pH and sour taste. It was reported that the addition of nitrite/nitrate, fermentation period and storage temperatures are effective on LAB growth (Zhao et al. 2011). Besides, LAB counts may also change according to food additives during storage period, amount, type and activity of starter cultures (Baytal 2023). Correspondingly, there may be differences in the number and activity of starter cultures.

Total Yeast and Mold Counts (TYM)

Some species of molds and yeast can affect color, aroma and odour whereas some of them cause deterioration (Şenol and Nazlı 1996, Yıldırım 1996). In the Turkish Food Codex Microbiological Criteria Regulation (TGK 2012) and TS 1070 (2002), maximum limits of total yeast and mold counts were approved as 10 in 1 g sample. Accordingly, 18 (60 %) of 30 samples are over the limit of TS 1070 Sucuk Standard (TS 1070 2002).

Mean total yeast and mold counts of previous studies by Şenol and Nazlı (1996), Günşen et al. (2001), Kök et al. (2007), Pehlivanoğlu et al. (2019) ; >5 log cfu/g and Öksüztepe et al. (2011) were reported as 4.72 log cfu/g, 3.28 log cfu/g, 3.00 log cfu/g, >5 log cfu/g, and 3.08 log cfu/g, respectively. Current results are lower than these counts.

In the first days of ripening, depending on the environmental aspects, mold and yeast counts increase rapidly and reach to 6 log cfu/g. Following days, along with the decreases in pH, water activity and redox potential value, mold and yeast counts decrease towards the end of ripening, and they become more concentrated against the exterior surface of sucuk (Tekinşen et al. 1982).

Total Coliform Bacteria Counts

Coliform group bacteria, a member of the family Enterobacteriaceae, are commonly an indicator of cross-contamination during the production process. They are capable of converting carbohydrate substrates into acid and may also reduce nitrate to nitrite and degrade the proteins (Yıldırım 1996). However, it was stated that coliform counts should not be high because they are correlated to being an indicator of probable hygiene and technological mistakes in final products (Nazlı 1995). Dominant microflora in traditional fermented meat products are highly related with the quality and the safety of the foodstuff.

In similar studies by Çon et al. (2002) stated mean coliform count as >6 log cfu/g, Öksüztepe et al. (2011) reported coliform counts ranged between 1.10- 2.6 log cfu/g, Pehlivanoğlu et al. (2015) indicated coliform counts in the range of <1 to 2.67 log cfu/g. Detected values are quite below the counts stated by previous researches. Turkish Food Codex Microbiological Criteria Regulation (TGK 2012) and TS 1070 (2002) limited the coliform counts to 10 in 1 g sample according to the most probable number method. Eventually, our samples have lower coliform counts than TS 1070 legislation (TS 1070 2002).

The presence of hygiene indicator microorganisms, especially coliforms, over a specific level in sucuk may be a result of inadequate or inaccurate ripening, the supply of raw materials in unhygienic conditions, or contamination during the process (Sancak et al. 1996).

***Staphylococcus aureus* Counts**

Öksüztepe et al. (2011) and Pehlivanoğlu et al. (2015) reported *S. aureus* counts of sucuk samples as 3.99 log cfu/g and 4.84 log cfu/g, respectively. Our current results are lower than these values.

According to the Turkish Food Codex Microbiological Criteria Regulation (TGK 2012) and TS 1070 (2002), the maximum number of *S. aureus* should be 100 in 1 g sample. All counts are below the legal limit in force. It was also determined that none of the sucuk samples contained *E. coli, Salmonella* spp. or *Listeria monocytogenes*.

CONCLUSIONS

The present study was aimed to determine the physicochemical and microbiological quality characteristics of the world-famous Afyon sucuk and its compliance with Communiqué on Turkish Food Codex Meat and Meat Products, Turkish Food Codex Microbiological Criteria Regulation (TGK 2012) and TS 1070 Sucuk Standard (TS 2002).

In terms of pH, protein content and fat content; 83.3%, 26.66% and 16.66% of current sucuk samples did not comply with Communiqué on Turkish Food Codex Meat and Meat Products, respectively. On the other hand, ash and salt contents are in accordance with legal regulations.

Total aerobic mesophilic bacteria and total yeast/ mold counts of 60% of sucuk samples are exceeding the legal limits in TS 1070 Sucuk Standard. Besides that, total *Coliforms*, *S.aureus*, *E.coli, Salmonella spp.* and *L.monocytogenes* counts are within the limits in Turkish Food Codex Meat and Meat Products, Turkish Food Codex Microbiological Criteria Regulation (TGK 2012and TS 1070 Sucuk Standard (TS 2002).

Especially, the physicochemical quality parameters were determined to be over the specified values in force in this research. Therefore, tightening the inspection mechanism must be in process. The absence of some of the physicochemical and microbiological quality criteria in related regulations rarifies standard production. Additionally, HACCP and GMP requirements need to be followed throughout the entire production process to ensure that products are safe and meet microbiologic standards.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: RŞ and HY contributed to the project idea, design and execution of the study. HY, GA, ÇA and AJD contributed to the acquisition of data. RŞ, HY and GA analysed the data. GA, ÇA and AJD drafted and wrote the manuscript. RŞ reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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CASE REPORT

The Effect of Platelet-Rich Fibrin (PRF) on Wound Healing in a Dog with Comorbidities

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ABSTRACT

Platelet-rich fibrin (PRF) is an autogenous material produced from the patient's own platelets, used to improve wound healing and tissue regeneration. In this case report, the effect of PRF on wound healing is investigated in a dog with tissue damage associated with infection by different pathogens (Anaplasma, Canine Coronavirus, Pneumonia, and Citruvite Crystals). PRF was locally applied to the wound area for 21 days, and it was observed that the wound area and the underlying bone tissue healed without necrotic tissue. It has been concluded that PRF accelerates wound healing and tissue regeneration in material loss tissue injuries accompanied by a multifactorial disease in this case.

Keywords: Anaplasma, canine, coronavirus , multifactorial disease, platelet-rich fibrin

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Komorbidli bir Köpekte Plateletten Zengin Fibrin'in (PRF) Yara İyileşmesi Üzerindeki Etkisi

ÖZ

Platelet zengin fibrin (PRF) hastanın kendi trombositlerinden üretilen otojen bir materyaldir ve yara iyileşmesini ve doku rejenerasyonunu iyileştirmek için kullanılır. Bu vakada, farklı patojenlerle (Anaplasma, Canine Coronavirus, Pnömoni ve Sitrüvit Kristalleri) ilişkili doku hasarı olan bir köpekte PRF'nin yara iyileşmesi üzerindeki etkisi araştırılmıştır. PRF, yara bölgesine lokal olarak 21 gün boyunca uygulanmış ve yara bölgesi ile altta yatan kemik dokuda herhangi bir nekrotik doku olmadan iyileştiği gözlemlenmiştir. Bu durumda PRF'nin, multifaktöriyel bir hastalık tablosu ile birlikte oluşan doku kayıplı yaralanmalarda yara iyileşmesini ve doku rejenerasyonunu hızlandırdığı sonucuna varılmıştır.

Anahtar kelimeler: Anaplazma, köpek, koronavirus, multifaktöriyel hastalık, platelet zengin fibrin

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INTRODUCTION

The wound is called to disrupt tissue integrity in the skin, subcutaneous tissues, muscles, and bones (Campanholi et al., 2022). The wound-healing process involves a complex but important sequence of interrelated stages, including hemostasis/inflammation, proliferation, and remodeling (Wang et al., 2018). Adequate wound healing in animals and humans depends on various variables, including circulation, wound size, width between wound edges, mobility, infection, and underlying tissue type (Iacopetti et al., 2012). Due to its frequent occurrence in different breeds and species of animals, wound healing is a comprehensive area of study in veterinary medicine. In this context, other treatments are being developed to accelerate the healing process and reduce the risk of secondary infection (Porsani et al., 2016).

Platelet-rich fibrin (PRF) is an autogenous material produced from the patient's own platelets, used to improve wound healing and tissue regeneration (Fan et al., 2020). Autologous platelet therapy became popular in the 1990s with the use of platelet-rich plasma (PRP). However, it has been said to have problems, including low effectiveness, the need for thrombin from animals for clotting, and differences between individuals (Karimi and Rockwell, 2019). In contrast, PRF does not require any additional substances during preparation, does not contain anticoagulation factors known to inhibit wound healing, guides natural clot formation, and supports growth factors and stem cell formation, among other advantages (Fan et al., 2020). These advantages led to the term PRF being first coined by Joseph Choukroun in 2001 (Choukroun et al., 2001).

PRF forms a three-dimensional fibrin network containing live cells that can serve as a scaffold in the early stages of wound healing. At this stage, platelets interact with the fibrin matrix to create a hemostatic plug and stimulate cell migration and proliferation (Pitzurra et al., 2020). The interaction between these cells and the fibrin matrix simulates the slow release of growth factors which can result in better wound healing in the early stages of this process (Davis et al., 2014).

CASE HISTORY

The material of the study was a 3-year-old mixed breed stray dog brought to the Milas Veterinary Faculty of Muğla Sıtkı Koçman University. During the initial examination, an open wound covering the left metacarpal and phalanx regions and reaching the depth of the bone tissue was observed. Subsequently, during a clinical examination, it was determined that the rectal body temperature value, mucous membrane colour, capillary refill time, and lung sounds in auscultation were normal. It was also found that the patient's vital signs were stable, and there was no orthopedic problem during the orthopedic examination. Open wound treatment with PRF was started immediately on the first day of admission.

During the clinical examination after the patient's general condition deteriorated in the following week, a rectal body temperature of 40.2 °C was measured, and reactive lymph nodes were palpable. The patient developed anorexia and weakness two days later. Hematological measurements revealed a decrease in lymphocytes, eosinophils and platelets. Suspecting an infection caused by blood parasites, a rapid diagnostic test kit named 'Vet Diagnostix Ehrichia + Lyme + Anaplasma + Heartworm Ag' was used (Canivet Tick-4 Combo Test, a lateral flow immunochromatographic test for qualitative detection of *Ehrlichia canis* antibodies (EHR), *Borrelia burgdorferi* antibodies (LYM) and *Anaplasma* spp. (ANA) antibodies and Heartworm (CHW) antigens in dogs' serum, plasma, and whole blood samples). The result of this test showed that *Anaplasma* spp. was positive and treatment was started accordingly. On the same day, another rapid test kit named 'VET Diagnostix Parvo (CPV) Ag + Corona (CCV) Ag + *Giardia*' showed that Canine Coronavirus was positive (Canivet CPV-CCV-*Giardia* Ag Combo Test, a lateral flow immunochromatographic test for differential diagnosis of canine parvovirus antigen (CPV Ag), canine coronavirus antigen (CCV Ag) and *Giardia lamblia* antigen (Giardia Ag) in the dog's feces or vomit sample). In addition, dysuria and stranguria were observed in the patient, and an abdominal ultrasound examination was performed. This examination revealed thickening of the bladder walls with accompanying cystitis, as well as the presence of bleeding and struvite crystals in the sediment, and treatment was initiated for this condition as well.

The hematological evaluation was conducted in the following days to monitor the patient's condition, lymphopenia, neutropenia, monocytopenia, and thrombocytopenia were detected. An increased respiratory rate, loss of appetite, and a high fever were observed. Pathological sounds were detected in the lung auscultation, and treatment was started. As a result of this picture, a pneumonia diagnosis was made. In the following days, a severe anemia picture accompanied by thrombocytopenia developed in the patient, and blood transfusion was performed with the help of another donor. On the second day following the transfusion, the patient passed away.

Preparation and Application of PRF

After the clinical examination, mechanical cleaning of the wound was performed. To regain the vitality of the wound, irrigation was carried out with isotonic 0.9% NaCl solutions. The purification of PRF from the blood samples was done according to the method of Dohan et al. (2006) with some modifications. For

preparing PRF, a blood sample (15ml) was collected from the cephalic vein of a clinically healthy dog into red blood tubes from another healthy dog. The tube was centrifuged at 3000 rpm for 10 minutes.

After the centrifugation, three layers were observed in the blood tube (Figure 1). On the bottom layer, which is not used, contains the part rich in red blood cells. The middle layer includes the clotting PRF layer, which is rich in platelets. The top layer consists of acellular plasma (Fan et al., 2020). A fibrin clot taken from the middle part with sterile forceps (Figure 2) was homogenized by mixing it with 10 grammes of vaseline pomade in sterile sample containers without delay for its application to the wound area.

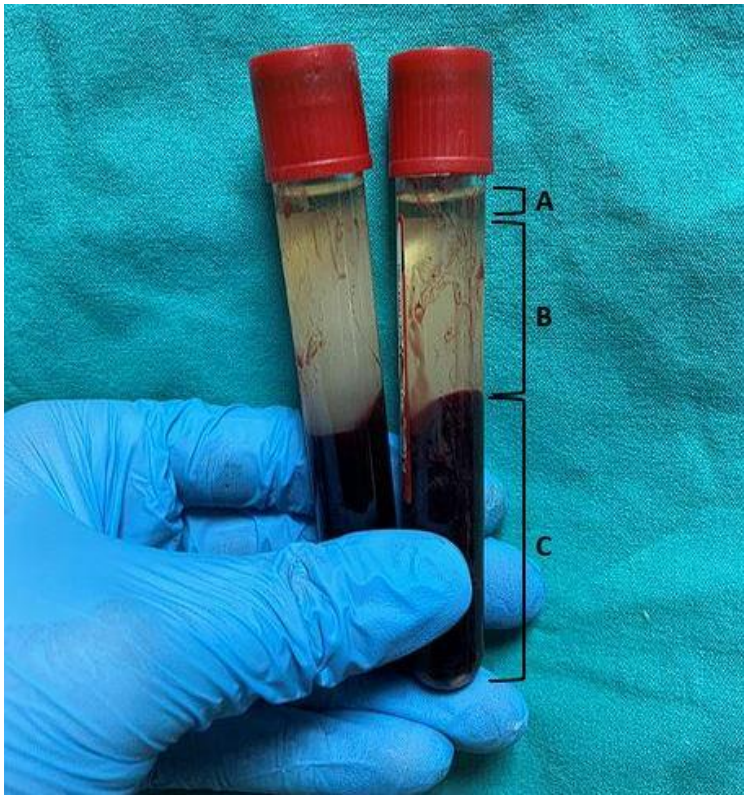

Figure 1: The three layers formed in the blood tube after the centrifugation process are as follows: **Figure 1A:** The acellular plasma portion, **Figure 1B:** The clotted PRF layer, **Figure 1C:** The layer rich in red blood cells.

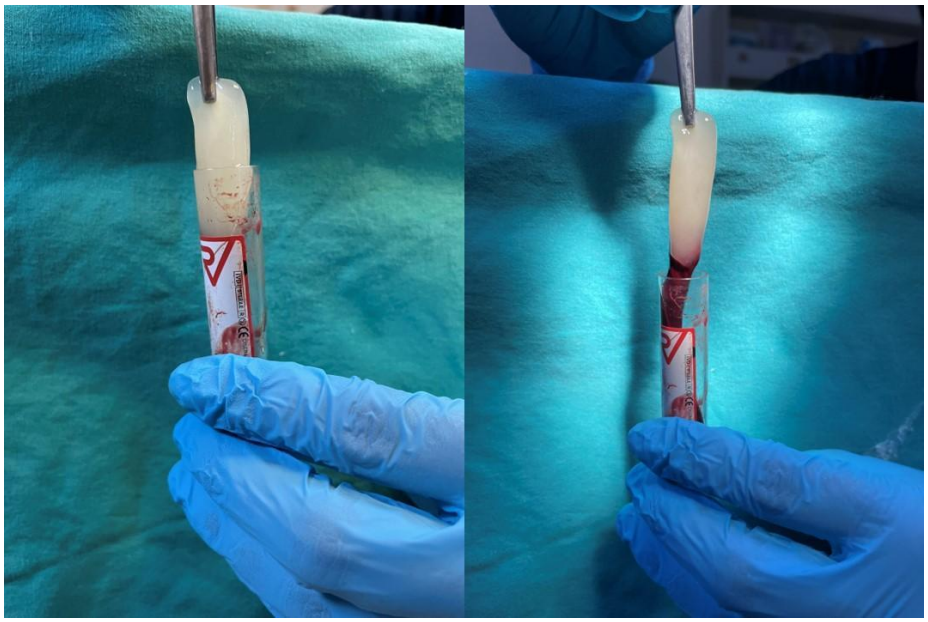

Figure 2: The fibrin clot obtained from the middle section with the help of sterile forceps.

The homogeneous mixture obtained was applied to the wound area homogeneously and then covered with sterile materials (Figure 3). The patient was also given systemic antibiotics (Synulox,

Amoxicillin/Clavulanate Acid 15mg/kg) and systemic analgesic treatments (Bavet Meloxicam, Meloxicam 0.2mg/kg) during the same period. The wound was covered with the same dressing bandage by repeating the same procedures every day for the first week and three times a week for the next two weeks, 21 days (Soares et al., 2021).

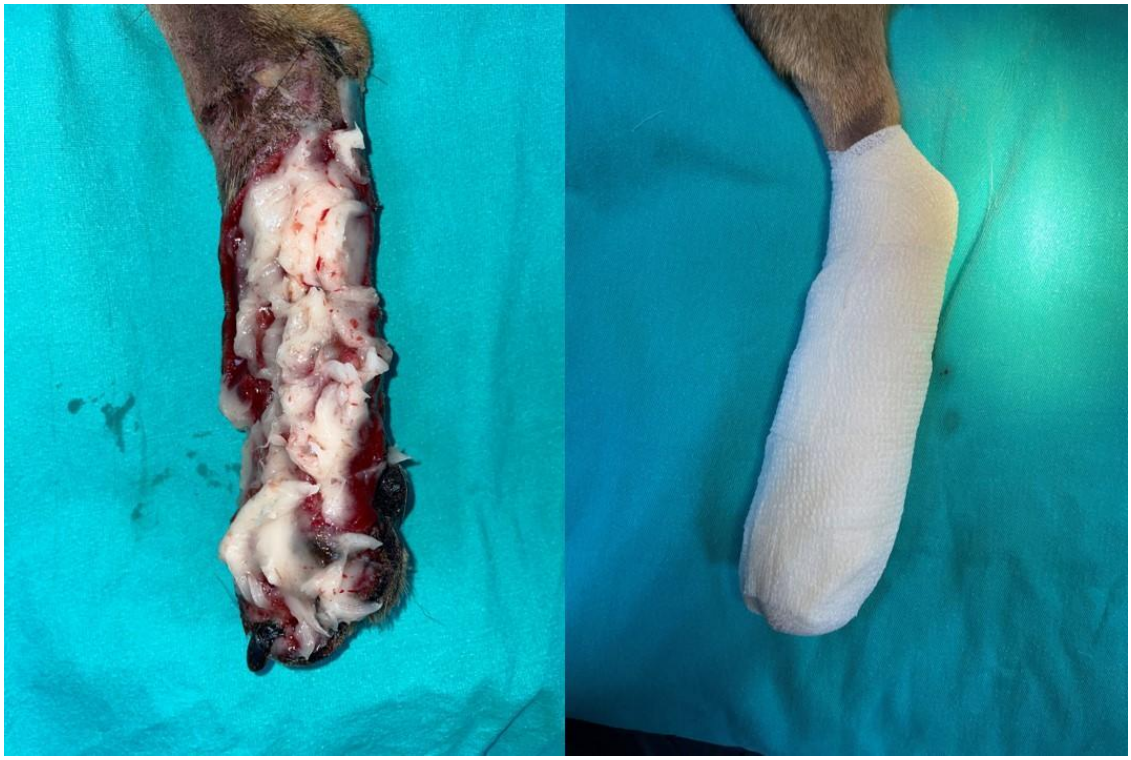

Figure 3: Application of PRF mixed with vaseline ointment to the wound area and dressing.

In this case report, the effect of PRF on wound healing is investigated in a dog with tissue damage associated with infection by different pathogens.

Comorbidity Management: Treatment Strategies for Other Diseases

The term comorbidity refers to the presence of any additional disease or disease in an individual with a particular disease that is not a complication of that main disease (Magyari and Sorensen, 2020). In this case report, the dog was referred to as a dog with comorbidities' due to its multifactorial disease profile. For the treatment of identified comorbidity pathogens; doxycycline 5-10 mg/kg/12 hours PO and enrofloxacin 5 mg/kg/12 hours SC were used, and fluid therapy, B vitamin complex, and amino acid solutions were used IV for supportive treatment. The sediment and crystal appearance seen on ultrasound examination of the bladder were determined to be struvite crystals on microscopic examination. Therefore, a preparation containing vitamin C was included in the prescription. Lavage was applied by resisting the bladder with a sterile dog urinary catheter 'Buster' brand, and thus, sediment and crystals within the bladder were removed. An unaffected male dog was used as a donor for the blood transfusion performed on the patient. A blood transfusion was performed at 20 ml/kg/4 hours.

DISCUSSION

Wound cases have found a comprehensive field of study in the veterinary medicine area from the past to the present due to their frequent occurrence in different animals. Various treatment methods have been tried to speed up the healing process and reduce the risk of secondary infections, such as platelet concentrates like PRF, which are used for regenerative procedures in various medical fields including dentistry, reconstructive surgery, plastic surgery, and dermatology (Porsani et al., 2016; Soares et al., 2021). In this case, the effect of PRF on wound healing in a dog with multifactorial disease has been examined. PRF has been reported as an effective treatment procedure in wound healing as a primary and complementary technique due to the long-term release of fibrin matrix, cellular components, and growth factors (Karimi and Rockwell, 2019).

Wound healing occurs through a series of events, starting with hemostasis and followed by inflammatory, proliferative, and finally remodeling phases. Hemostasis begins with platelet aggregation and continues with thrombus formation. The inflammatory phase starts with the accumulation of neutrophils and macrophages at the wound site within 24-48 hours (Scopelliti et al., 2022). The proliferative phase encompasses re-epithelialization, angiogenesis, collagen accumulation, and granulation tissue formation. Cytokines such as interferon (IFN) and transforming growth factor (TGF) are stimulated to synthesize factors such as collagen and fibronectin,

which facilitate fibroblast proliferation, the closure of tissue gaps, and the restoration of mechanical strength. Epidermal growth factor (EGF), fibroblast growth factor (FGF), and TGF stimulate keratinocyte proliferation and re-epithelialization by promoting migration on the wound bed. Simultaneously, angiogenesis is induced by various growth factors such as vascular endothelial growth factor (VEGF), FGF, and platelet-derived growth factor (PDGF). Neo-angiogenesis is further facilitated by releasing proteolytic enzymes and metalloproteinases (MMP) by endothelial cells that degrade the basement membrane and surrounding tissues (Scopelliti et al., 2022). PRF is a three-dimensional structure containing 97% platelets and >50% leukocytes, and it contains cytokines and growth factors (TGF-β1, IGF-1 and 2, PDGF, VEGF, IL-1, 4, and 6) that play a role in tissue regeneration and wound healing (Kızıltoprak, 2019). It has been stated that PRF positively affects and accelerates wound healing thanks to these components (Lektemur Alpan and Torumtay Cin, 2020). Lundquist et al. (2008) emphasized in their study the importance of fibrinogen factors contained in PRF for wound healing and tissue regeneration. Clipet et al. (2012) stated in their study that growth factors found in PRF induce cell viability and proliferation differentiation. In an in vivo study conducted by Roy and colleagues (2011), it was reported that PRF stimulated angiogenesis and accelerated wound healing at the end of 14 days (Roy et al., 2011). In a study conducted by Tunali et al. (2013), it was reported that PRF accelerates wound healing in oral mucosal injuries in rabbits. Desai et al. (2013) followed the wound healing process with the PRF obtained after applying a centrifuge process for 10 minutes at 3000rpm in a 30-year-old individual. They emphasized that PRF is a successful innovative technique in wound healing. In line with the studies conducted, the effects of PRF on wound healing have been examined in this case, and it has been observed that the wound area and the underlying bone tissue healed without any necrotic tissue.

What sets this condition apart from other illnesses is a multifactorial disease profile. Among these diseases, is Anaplasmosis, which is caused by the obligatory intracellular pathogen *Anaplasma* spp. transmitted by ticks and belonging to the Anaplasmataceae family, is of great importance (Dahmani et al., 2017). In a recent study, it was shown that *Anaplasma* spp. infection increases the activity of interferon and neutrophil chemotaxis pathways in the skin, while interestingly decreasing the expression of genes involved in extracellular matrix (ECM) organization and wound healing responses (Underwood et al., 2022). However, in this case, it was observed that there was a significant macroscopic improvement in the open wound of the dog infected with *Anaplasma* spp. after 21 days. Therefore, further studies are needed to clarify this situation more clearly.

During the 21-days for wound healing, PRF was locally applied to the wound area and no signs of infection were detected during the application. During the first week, when dressing and PRF application were repeated daily, the wound tissue was constantly monitored . At the end of the week, a significant demarcation area was observed on the wound lips, and rapid wound healing was seen. In the following two-week period, dressing and PRF application were repeated three times a week. Throughout the 21-days, no signs of infection, dead tissue, or aggressive wound lips were found in the wound area. On the contrary, it was observed that the boundaries of the wound area progressed significantly and the underlying bone tissue healed without any necrotic tissue formation (Figure 4).

Figure 4: The macroscopic appearances of the wound area from day zero to day twenty-one.

At the end of the 21-day treatment, it was determined that healing was still ongoing at the site of the wound, however, the patient, who had some systemic diseases and was infected with different pathogens, passed away in the following period.

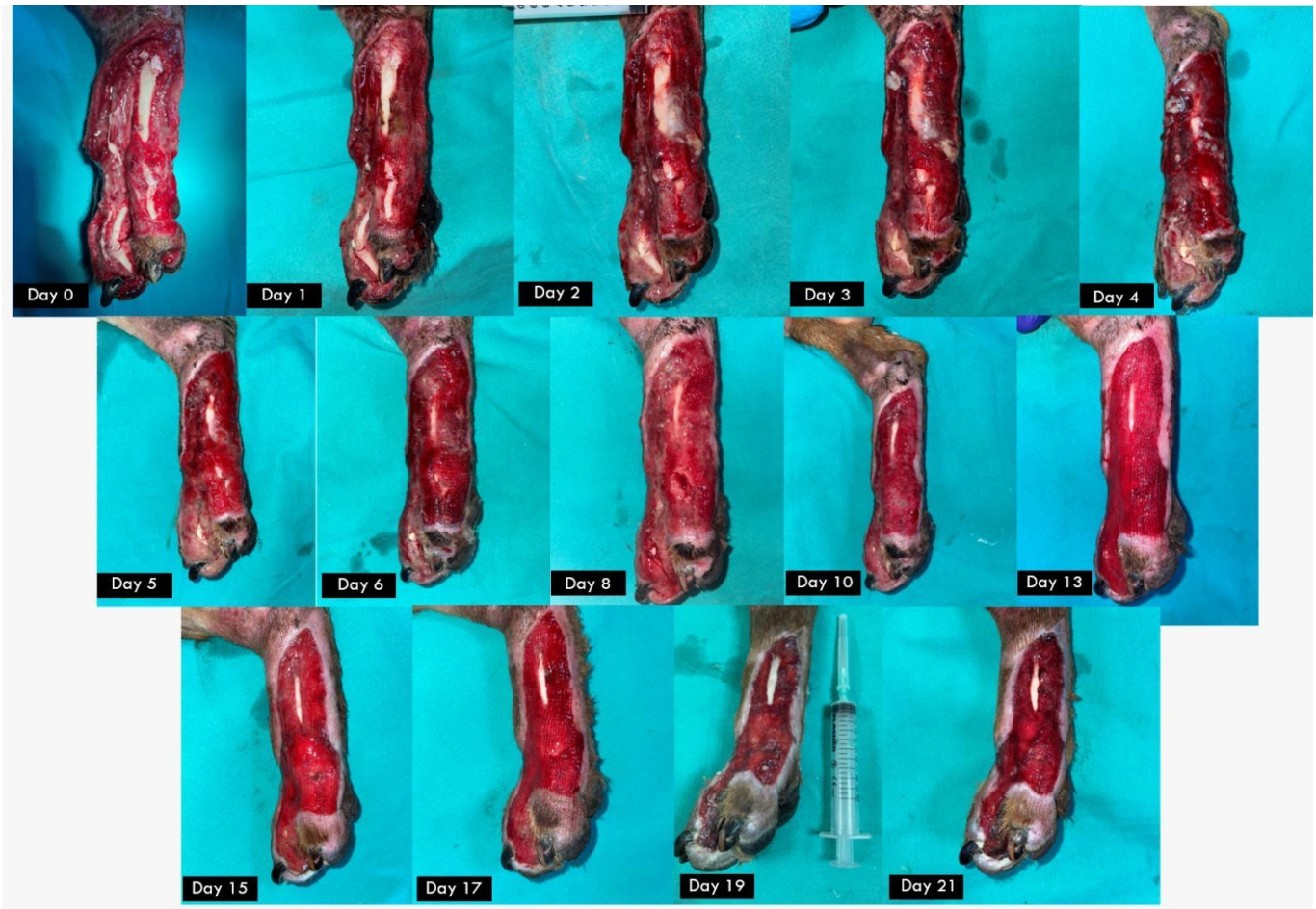

Figure 4: The macroscopic appearances of the wound area from day zero to day twenty-one.
At the end of the 21-day treatment, it was determined that healing was still ongoing at the site of the wound, however, the patient, who had some systemic diseases and was infected with different pathogens, passed away in the following period.

CONCLUSION

As a result, it has been concluded that PRF accelerates wound healing and tissue regeneration in material loss tissue injuries accompanied by a multifactorial disease presentation in this case. However, further studies are needed to demonstrate the effectiveness of PRF in large material loss open wounds.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: All authors contributed to the project idea, designing and conducting the study, obtaining data, analyzing data, drafting and writing the manuscript, and critically reviewing the manuscript. All authors have read and approved the final manuscript.

Ethical approval: This study was carried out in Muğla Sıtkı Koçman University Milas Veterinary Faculty Training and Practice Hospital. This study was approved by the Ethics Committee of Muğla Sıtkı Koçman University Experimental Animals Application and Research Center Animal Experiments Local Ethics Committee (MUDEM-HADYEK, Ref No: E-40051172-100-622678, Date: 21/06/2023)

Explanation: Presented as an oral presentation at the 8th International Congress on Advances in Veterinary Sciences and Techniques (ICAVST) (2023).

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CASE REPORT

Multi-Etiological Abortion due to *Campylobacter* spp. and *Chlamydia abortus* in a Sheep

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ABSTRACT

In this case, multi-etiologic abortion due to *Campylobacter* spp. and *Chlamydia abortus* (*C. abortus*) was reported from an aborted sheep fetus sent to Konya Veterinary Control Institute (KVKE) from a sheep farm operating in Aksaray province in 2020. The presence of *Campylobacter* spp. was determined by the bacterial isolation method. *C. abortus* was identified by qPCR and immunohistochemistry (IHC) methods. In this study, it was aimed to indicate that multi-etiological abortions involving multiple factors should be taken into consideration in the fight against sheep abortions, that it would be appropriate to use a simultaneous multidisciplinary approach in diagnosis to identify abortion factors, and that it could contribute to a more effective fight against abortions.

Keywords: *Campylobacter* spp., *Chlamydia abortus*, Immunohistochemistry, Multi-etiological abortion, PCR.

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Bir Koyunda *Campylobacter* spp. ve *Chlamydia abortus* Tarafından Oluşturulan Multi-Etiyolojik Abort

ÖZ

Bu vakada, Konya Veteriner Kontrol Enstitüsü'ne (KVKE) 2020 yılında, Aksaray ilinde faaliyet gösteren bir koyun işletmesinden gönderilen aborte koyun fetusunda *Campylobacter* spp. ve *Chlamydia abortus* (*C. abortus*) etkenlerine bağlı multi-etiyolojik abort belirlendi. *Campylobacter* spp. varlığı bakteriyel izolasyon yöntemi ile belirlendi. *C. abortus* ise qPZR ve immunohistokimya (İHK) yöntemleri ile tanımlandı. Bu çalışmayla, koyun abortlarıyla mücadelede birden çok etkenin karıştığı multi-etiyolojik abortların dikkate alınması, abort etkenlerini tespit etmek için teşhiste eş zamanlı multidisipliner bir yaklaşımın kullanılmasının uygun olacağı ve abortlarla daha etkin bir mücadeleye katkıda bulunulabileceğini belirtmek amaçlanmıştır.

Anahtar kelimeler: *Campylobacter* spp., *Chlamydia abortus*, İmmunohistokimya, Multi-etiyolojik abort, PCR.

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INTRODUCTION

Pregnancy and birth rates are relatively high in sheep during the mating period. To maintain profitability in ovine breeding, the aim is to have at least one offspring per year. There are significant problems in the birth of healthy lambs in sheep. The main problem affecting this goal in both ovine and bovine animals is abortion, which can cause severe economic losses (Erdem and Sarıbay 2019). Abortion factors that can emerge at any stage of pregnancy are classified as infectious or noninfectious causes. Noninfectious causes include care and nutritional disorders, environmental conditions, and misuse of hormones and drugs (Ay 2017). Infectious causes, which play a much more significant role in the aetiology of abortion, include viral, parasitic, bacterial, and fungal agents. Infectious abortions are often herd-based rather than sporadic cases. Despite the differences in prevalence among countries, the most significant infectious abortion agents in sheep of Türkiye are *Brucella melitensis* and *Chlamydia abortus* (*C. abortus*), *Campylobacter fetus subsp. fetus*, *Salmonella abortusovis*, *Akabane disease virus (Arbovirus)*, *Border disease virus* (*Pestivirus*), *Bluetongue disease virus (Orbivirus)*, *Coxiella burnetii*, *Toxoplasma gondii*, and *Neospora caninum*. Some of these pathogens are zoonotic agents that cause miscarriage and stillbirth in domestic animals and humans (Gulaydın et al. 2023).

Campylobacter spp. colonize the intestines of sheep and cattle. It can cause sporadic abortions in both species (Lastovica and Allos 2008). *Campylobacter* spp. are recognized as critical causative agents of ovine abortions in Türkiye and worldwide. *Chlamydiae*, an obligate intracellular and gram-negative bacterium, causes many diseases in cattle, sheep, goats, pigs, and humans. *Chlamydiae* causes kerato-conjunctivitis, pneumonia, enteritis, hepatitis, mastitis, polyarthritis, sporadic encephalomyelitis, vaginitis, endometritis, fertility problems, and abortion in ruminants (Otter et al. 2003).

This study aimed to describe a case of multi-ethiologic abortion due to *Campylobacter* spp. and *C. abortus* diagnosed in a sheep abortion and to draw attention to multi-ethiologic abortions in the fight against abortions.

CASE HISTORY

Informed consent was obtained from the flock owner for this case report. In 2020, an aborted fetus from a sheep flock in Aksaray province was submitted to the KVCI. According to the information provided by the owner of the herd, there was a small number of abortions in each parturition period in the herd, but the abortion rate reached 30-35% in 2020. It was reported that only the vaccines against *Brucella melitensis* Rev-1 and *Peste Des Petits Ruminants Virus* (PPRV) were applied in the herd within the framework of the programmed vaccinations of the official institutions. The aborted fetus was necropsied, and stomach contents, lung, liver, heart, and umbilical cord were taken for laboratory studies.

Macroscopically, autolytic changes were detected in the aborted sheep fetus at the first examination. In addition, typical macroscopic findings were not observed during necropsy, but the fetal stomach contents were egg-white in colour and consistent. (Figure 1A).

For bacteriological examination, fetal stomach contents and fetal liver tissues were inoculated on *Campylobacter* agar (CM0689, UK) enriched with 7% sheep blood by adding *Campylobacter* selective supplement (Oxoid, Skirrow, SR0069E, UK). Then, the mixture was incubated for 2-3 days at 37 °C in a 10% CO_2 (microaerophilic) oven (Thermo Heracell 150, Germany). The colonies observed after incubation were classified according to their morphological characteristics by Gram staining (Martin et al. 2002). As a result of bacteriologic investigations, *Campylobacter* spp. was isolated from the stomach contents and liver of the aborted fetus. A Gram-stained image of the isolated *Campylobacter* spp. is presented in Figure 1B.

Chlamydiae, which are obligate intracellular bacteria, require a living organism for isolation and multiplication. Therefore, they cannot be isolated using the classical bacteriological culture method. For this purpose, cell culture or inoculation of embryonic chicken eggs are the preferred standard methods for isolating the pathogen. However, these methods may not be considered practical because they require a suitable laboratory environment and involve a laborious process (Woah 2018). In this case, qPCR and IHC methods were used to diagnose *C. abortus*.

The lung, liver, heart, and umbilical cord were frozen at -20 °C after necropsy for DNA extraction. Tissues were then dissected. DNA was extracted from the supernatant after centrifugation via an automatic extraction device (QIAcube, Qiagen, Germany) according to the manufacturer's protocol (IndiSpin Pathogen Kit, Indical Bioscience, Germany). qPCR analysis was performed with Qiagen Rotor-Gene Q (Qiagen, Germany) according to the kit protocol using a primer/probe set targeting the *ompA* gene (Pantchev et al. 2009) and a LightCycler 480 Probe Master Kit (Roche, USA) as indicated in Table 1. qPCR analysis was used to detect *C. abortus* DNA in fetal tissues.

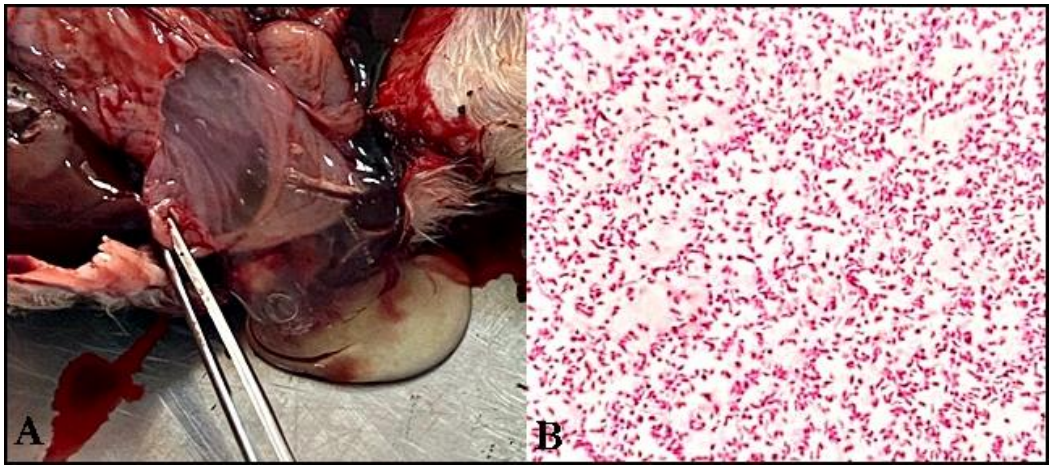

Figure 1: A. Fetal stomach contents. B. Gram stained image of spiral shaped *Campylobacter* spp. bacteria. Original magnification. 1000X.

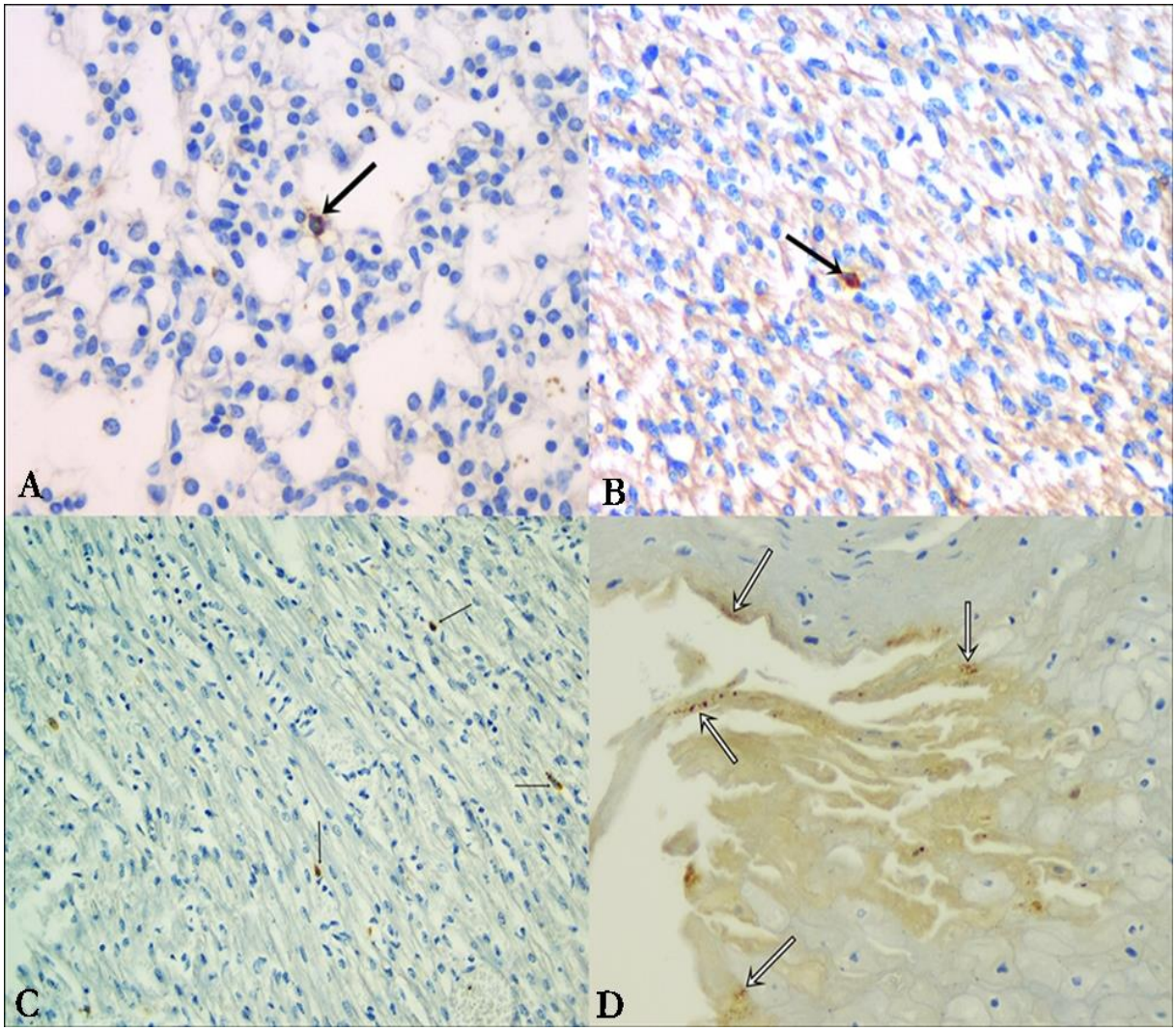

Figure 2: A. Lung. *Chlamydia* spp. positivity in alveolar macrophages (black arrow). Indirect IHC. 400X. B. Liver. *Chlamydia* spp. positivity in macrophages (black arrow). Indirect IHC. 400X. C. Heart. *Chlamydia* spp. positivity in inflammatory infiltrates (black arrows). Indirect IHC. 200X. D. Umbilical cord. *Chlamydia* spp. positivity (white arrows) in epithelial cells. Indirect IHC. 200X.

Table 1. Primer/probe sets are used in PCR.

Abort Agent	Diagnostic Method	Primer/Probe Index	Amplicon Size (bp)	Reference
Akabane Disease Virus	One-Step RT-PCR	AKSF19: 5'-TAA CTA CGC ATT GCA ATG GC-3' AKSR740: 5'- TAA GCT TAG ATC TGG ATA CC-3'	-	Akashi et al. 1999
Border Disease Virus	One-Step RT-PCR	PBD1: 5′-TCGTGGTGAGATCCCTGAG-3′ PBD2: 5′-GCAGAGATTTTTTATACTAGCCTATRC-3′	-	Vilbek and Paton 2000
Bluetongue Disease Virus	RT-PCR	F:5'-TGGAYAAAGCRATGTCAAA-3' R:5'-ACRTCATCACGAAACGCTTC-3' P:5'FAM-ARGCTGCATTCGCATCGTACGC-3' BHQ1	-	Hofmann et al. 2008
C. abortus	qPCR	F: 5'-GCAACTGACACTAAGTCGGCTACA-3' R: 5'-ACAAGCATGTTCAATCGATAAGAGA-3' P:(FAM-AAATACCACGAATGGCAAGTTGGTTTAGCG-TAMRA)	82	Pantchev et al. 2009
Neospora caninum	Nested PCR	JB1: 5′AGGAGGAGAAGTCGTAAGG3′ JB2: 5′GAGCCAAGACATCCATTGC3′	500	Barratt et al. 2008
Peste Des Petits Ruminants Virus (PPRV)	One-Step RT-PCR	NP3: 5'-GTCTCGGAAATCGCCTCACAGACT-3' NP4: 5'-CCTCCTCCTGGTCCTCCAGAATCT-3'	-	Couacy-Hymann et al. 2002
Toxoplasma gondii	qPCR	F: 5'-GGAGGACTGGCAACCTGGTGTCG-3' R: 5'-TTGTTTCACCCGGACCGTTTAGCAG-3' P-1: 5'-ACGGGCGAGTAGCACCTGAGGAGAT-3' P-2: 5'-CGGAAATAGAAAGCCATGAGGCACTCC-3'	126	Costa et al. 2000
Brucella spp.	Bacteriological Culture	-	-	Alton et al. 1988
Campylobacter spp.	Bacteriological Culture	-	-	Martin et al. 2002
Listeria spp.	Bacteriological Culture	-	-	Jinneman et al. 2003
Salmonella spp.	Bacteriological Culture	-	-	Woah 2022

For differential diagnosis, bacteriological cultures were performed from stomach contents and lung, liver, and heart samples to detect the presence of other microaerophilic and aerobic bacterial abortion agents (*Brucella* spp., *Listeria* spp., and *Salmonella* spp.) other than *Campylobacter* spp. Molecular tests were performed to investigate the presence of *Akabane disease*, *Border disease*, *Bluetongue disease viruses*, *Peste Des Petits Ruminants Virus* (PPRV), *Toxoplasma gondii*, and *Neospora caninum* (Table 1). Bacteriological culture and molecular analyses of fetal tissues were negative for *Brucella* spp., *Salmonella* spp., *Listeria* spp., *Akabane virus*, *Border disease virus*, *Bluetongue virus, PPRV*, *Neospora caninum*, and *Toxoplasma gondii.*

Tissue samples taken after necropsy and fixed in 10% buffered formalin were processed according to routine methods and blocked in paraffin. Then, five µm thick sections from the tissue blocks were subjected to IHC staining. Immunohistochemistry was performed on 4-5 µm paraffin sections on poly-L-lysine slides (Isotherm, Türkiye). Staining was then performed by biotinylated indirect IHC staining on a Ventana Benchmark XT using 125 µl of 1/200 diluted *Chlamydiaceae* specific mouse monoclonal

antibody (Cat. No. ACI-P, Progen Biotechnik GmbH, Germany) for each specimen according to the manufacturer's procedure (Ref. No. 760-500, UltraVIEW Universal DAB Detection Kit, USA). All sections of the tissues were examined under a light microscope. *Chlamydia* spp. IHC positive control preparations from the Pathology Laboratory of the Faculty of Veterinary Medicine, University of Zurich, Switzerland, were used. Immunohistochemical staining with sterile PBS was used as a negative control instead of the primary antibody in sections. As a result of IHC staining, granular or homogeneous brown staining with cellular association on a blue background was considered positive. *Chlamydia* spp. immunopositivity was detected in the lungs, liver, heart, and umbilical cord of the aborted fetuses. *Chlamydial* antigens were localized to alveolar macrophages in the lung (Figure 2A), macrophages in the liver (Figure 2B), inflammatory infiltrates in the interstitial space in the heart (Figure 2C), and epithelial cells in the umbilical cord (Figure 2D).

DISCUSSION

Species belonging to the genus *Campylobacter* are responsible for many sheep abortions. Goats are more resistant to this infection, and the abortion rate is lower (Buyuk et al. 2011). *C. abortus* is also a critical abortion agent in Türkiye (Malal and Turkyilmaz 2021). In Türkiye, as in other countries, abortions continue due to the inability to obtain healthy offspring despite strict vaccination programs and severe economic losses that occur in animal husbandry. This situation suggests that in addition to abortions due to a single etiological agent, multiple etiological abortions may be more common than estimated. The introduction mentions that abortion cases in which different etiological agents are detected together have been reported in recent years (De Angelis et al. 2022; Ramo et al. 2022; Şevik et al. 2017a; Şevik et al. 2017b; Deniz and Oruc 2023). In the present study, a case of multi-etiological abortion caused by *Campylobacter* spp. and *C. abortus* in a sheep abortion was described.

Recent studies on abortion in Türkiye have shown positivity for different etiologic agents. Gulaydın et al. (2023) examined a total of 113 samples from 85 several sheep flocks by qPCR for bacterial abortion agents. They found that 42.8 % were positive for *C. abortus*, 25.7 % for *B. melitensis*, 14.2 % for *S. abortusovis*, 11.4 % for *Coxiella burnetii*, 2.8 % for *L. monocytogenes* and 2.8 % for *Campylobacter* spp. Although 42.8% *C. abortus* and 2.8 % *Campylobacter* spp. positivity was detected in the study of Gulaydin et al. (2023); it is understood that these results were obtained from different animals, and these agents were not multi-etiologically detected in a single aborted fetus. In this case, the presence of *Campylobacter* spp. and *C. abortus* in a single aborted fetus was demonstrated simultaneously with a multidisciplinary approach. Although 42.8 % C. abortus and 2.8 % *Campylobacter* spp. positivity was detected in the study by Gulaydin et al. (2023); it is understood that these results were obtained from different animals, and these agents were not detected multi-etiologically in a single aborted fetus. Sakmanoglu et al. (2019) investigated the epidemiology of pathogenic bacteria in 250 stomach contents of aborted fetuses of cattle, sheep, and goats from different regions of Türkiye by PCR and found 155 positive samples for bacterial agents. Of these positive samples, 58.88 % were found in sheep, 43.47 % in goats, and 67.15 % in cattle samples. The five most common bacteria associated with abortion were *Brucella melitensis* at 20.9 %, *B. abortus* at 5.2 %, *Leptospira* spp. at 13.6 %, *Campylobacter fetus* at 20.9 %, and *Coxiella burnetii* at 1.6 %. They did not find any positivity for *C. abortus* in the study. In addition, a multi-ethiologic situation in terms of bacterial agents was not reported in the positive results obtained in the study. In this case, the presence of *C. abortus* and *Campylobacter* spp. in a single aborted fetus was demonstrated simultaneously with a multidisciplinary approach.

Among the studies conducted worldwide, Ramo et al. (2022) in Spain reported both *Campylobacter* spp. and *C. abortus* positivity in 10 cases in a study conducted with qPCR in sheep and goat abortion materials. In addition, IHC staining results that were consistent with experimental and field studies investigating *Chlamydial* antigen localization were obtained (Livingstone et al. 2017).

In line with our studies and our report, domestic animals should not be ignored because they are not constantly exposed to a single abortion agent and because different etiological agents may play a role together and cause multiple etiological abortions. In an abortion study previously published (Deniz and Oruc 2023), *Brucella* factors were shown to accompany *Chlamydia* infection. In this regard, multi-etiological abortion cases due to *Chlamydia* should also be investigated, as should other multi-etiological miscarriages. Moreover, a multidisciplinary approach in which multiple factors are taken into consideration instead of a single etiological factor in abortion cases is necessary. Recognizing this and making a complete diagnosis will enable more accurate and better protection measures to be taken for animal health.

In our study, *Campylobacter* spp. was diagnosed by bacteriologic isolation, and *C. abortus* was diagnosed using qPCR and IHC methods. Therefore, it would be more beneficial to test many factors in abortion cases with multidisciplinary diagnostic methods.

In the studies by Tuzcu et al. (2010), Aydin et al. (2020), and Karakurt et al. (2020), multifocal necrotic hepatitis with a characteristic target board appearance in the liver was detected histopathologically in *Campylobacter* spp. positive abortions, whereas typical liver lesions were not observed in this case. It is thought that the reason why typical liver lesions were

not observed in this case may be due to the fact that the amount of bacteria was not at a level that caused typical lesions in the liver.

Many bacterial, viral, and parasitic agents found in Türkiye were investigated in the study, such as *Coxiella burnetii* and *Leptospira* spp. *Mycoplasma* spp. *C. pecorum, C. psittaci*, and *sheep pox virus* were not evaluated as abortion agents. Simultaneous screening for these abortion agents in the identification of multi-ethiologic abortion cases will help to identify abortion agents and control multi-ethiologic abortion.

CONCLUSION

In conclusion, *Campylobacter* spp. and *C. abortus* were detected in sheep offspring in Türkiye in this study, and it was found that similar abortion patterns should be considered in abortion control. It is known that *Brucella* species and *C. abortus* are the most common abortion pathogens in small ruminants in Türkiye. In abortion cases, *Brucella* is usually evaluated first, and in case of negativity, other agents are evaluated one by one according to their prevalence in sheep. In the case of positivity for any of these agents, a separate evaluation for other bacterial, viral, and parasitic agents is not usually performed. In this study, we tried to emphasize the importance of performing a multidisciplinary evaluation for bacterial, viral, and parasitic agents simultaneously in every abortion sample that comes to laboratories for diagnostic purposes. The knowledge of the existence of abortions with multifactorial aetiology will contribute to the diversification of the measures (preventive vaccination, treatment, etc.) to be taken in the fight against abortion factors. In addition, it was concluded that these infectious agents should be taken seriously and studied individually with a broader range of diagnostic methods rather than relying on limited methods of laboratory diagnosis.

Conflict of Interest: The authors declare that there are no conflicts of interest.

Authors' Contributions: Case review, evaluation of findings, images, manuscript writing and submission of the manuscript were performed by ID. The discussion section was organized and contributed to the discussion by EO.

Ethical Approval: "This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules." (Ref No: 701825, Date: 02/2024).

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