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Research article

Exploring the anti-proliferative and cytotoxic impact of doxycycline on C6 glioma cells

Firuze Unlu Bektas*[1], Mine Kucak[1], Ekin Bektas[1], Tolga Onturk[1], Muhammed Hamza Muslumanoglu[1]

[1] *Yildiz Technical University, Faculty of Arts & Science, Department of Molecular Biology and Genetics, 34220, Istanbul, Türkiye*

Abstract

Doxycycline is a member of the tetracycline group and is a bacteriostatic antibiotic. Therefore, it stops/slows down the reproduction and spread of pathogenic microorganisms and gives the immune system the necessary time to destroy them. In this study, cytotoxic, anti-proliferative, and apoptotic effects of doxycycline on the rat glioma cell line derived from *Rattus norvegicus* were observed. To show the cytotoxicity of doxycycline, MTT test was performed to obtain the IC_{50} value and the dosages of treatment were determined accordingly. With the colony formation test, it was observed that the determined doxycycline dosages reduced the colony formation ability of the single cells. Similarly, wound healing test also showed that doxycycline treatment reduced the ability of cells to migrate. A dose-dependent decrease in the cell number was detected by DAPI staining after doxycycline treatment and the expression levels of cancer related genes were shown by the RT-qPCR method. In conclusion, doxycycline was found to have anti-proliferative and cytotoxic effects in rat glioma cell line, and more comprehensive studies are needed before doxycycline can be used as a complementary agent in cancer treatment.

Keywords: *Cancer; cytotoxicity; doxycycline; proliferation; rat glioma; RT-qPCR*

1. Introduction

Antibiotics, which are amongst the most frequently used drug groups all over the world, have effects other than their antimicrobial effects (Dugray et al., 2001; Singh et al., 2021; Li et al., 2023; Nassar et al., 2023; Duncan et al., 2024; Han et al., 2024). For instance, doxycycline, a tetracycline group antibiotic, has an anti-tumor effect (Lamb et al., 2015; Zhang et al., 2017; Ghasemi and Ghasemi, 2022; Chan and Kamath, 2023; Choi et al., 2024; Mi et al., 2024). Doxycycline is a semi-synthetic antibiotic (Syapin et al., 2016) and was first approved by FDA in 1967. Doxycycline, which has two chemical forms, monohydrate, and hyclate, is synthetically produced from oxytetracycline (Feitosa et al., 2022). Being a bacteriostatic antibiotic, it shows its antimicrobial effect by inhibiting the protein synthesis of bacteria (Feitosa et al., 2022; Shutter and Akhondi, 2023). It binds allosterically to the 30S prokaryotic ribosomal subunit during protein synthesis, preventing the production of the bacteria's essential proteins (Brodersen et al., 2000; Warner et al., 2022). Doxycycline also prevents cell proliferation, induces apoptosis, inhibits matrix metalloproteinases (MMP) and prevents tumor-induced angiogenesis (Yang et al., 2022; Siregar et al., 2023; Wehrli et al., 2023). Gliomas constitute 74.6% of all malignant brain tumors (Souza et al., 2018). Survival of patients with glioblastoma multiforme (GBM) caused by aggressive glioma does not exceed 15 months (Swartz et al., 2014; Romanishin et al., 2024). The C6 cell line is the cell line that most closely resembles the human brain tumors mechanism, according to the comparison between eight rats and it is similar to human glioblastoma in terms of high mitotic index, parenchymal invasion and neo-angiogenesis (Grobben et al., 2002; Barth and

* Corresponding author.
E-mail address: firuze-26@hotmail.com (F. Unlu Bektas).
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Kaur, 2009; Pournajaf et al., 2024). Doxycycline hyclate is a promising agent for use in cancer treatment, as it is currently an FDA-approved drug and has a low cytotoxic effect. This study was conducted to show its effects on cancer cells, which has been a big problem for a long time. Cytotoxic, anti-proliferative and apoptotic effects of doxycycline on cells were observed using proliferation tests and gene expression tests, at the dosages determined by cell viability test, *in vitro*.

2. Materials and methods

2.1. Cell culture

C6 rat glioma cells (ATCC-CCL-107) were provided by Yildiz Technical University (Istanbul, Türkiye). Dulbecco's Modified Eagle Medium-High glucose (DMEM) (Sigma) supplemented with 1% penicillin/streptomycin (Capricorn) and 10% fetal bovine serum (FBS) (Thermo Fisher) was used to grow the cells. They were cultured at 37°C with 5% CO_2. Cell culture was limited to twenty-five passages (P25).

2.2. Cell viability assay

MTT 3-(4,5-dimethyl-thiazolyl 2,5-diphenyltetrazolium bromide) assay was used as previously described to show the viability of the cells after treatment (Kumar et al., 2018). C6 glioma cells (5×10^3) were plated in 96-well culture plates overnight. The experimental cells were treated with 20, 40, 60, 80, 120, and 160 µM concentrations of doxycycline hyclate (Across) for 48 hours. After 48 hours, 10 µL MTT (Goldbio) was added into the wells, and they were incubated at 37°C for 2 hours. 100 µL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, afterwards. The optical density was measured at 570 nm absorbance. GraphPad Prism 9.3.0 was used to calculate the IC_{50} value of doxycycline treatment.

2.3. Colony formation assay

Colony formation assay was used to determine the colony formation ability of a single cell after treatment (Franken et al., 2006). 250 cells per well were plated into 6-well culture plates overnight. After 24 hours, they were treated with 40 µM and 60 µM doxycycline and cultured for 12 days after 48 hours of treatment and the control group did not have any treatment. The medium of the cells was not changed during this period. The colonies were fixed and stained with Gram's crystal violet solution (Merck) after 12 days. ImageJ was used to count the colony number of cells.

2.4. DAPI staining

The same number of cells were plated in T25 flasks and cultured until 70% confluency. Experimental groups were treated with 40 µM and 60 µM doxycycline and they were cultured together with the control group for 48 hours. The cells were washed with 1X PBS (Wisent) and ice-cold methanol (Merck) was used to fixed the cells for 10 minutes. After fixing, the nucleus was stained using an 850 nm DAPI satin solution (Cayman) and protected from light. DAPI solution was removed afterwards, and the cells were washed with 1X PBS. They were captured under a fluorescent microscope (Zeiss) from 5 different areas of the flask. The cell numbers were counted with the image processing program ImageJ.

2.5. Wound-healing assay

Wound healing assay was applied as previously mentioned to observe the changes in cell migration in wound-created cells depending on the treatment (Jonkman et al., 2014). 24-well culture plates were used to plate (5×10^4) cells and the cells were cultured until confluency. A wound was made using a 100-µL sterile pipette tip. The cells were then cultured with 40 µM and 60 µM doxycycline treatment and the control group did not have any treatment. Images of the cells were captured at 0, 24, and 48 hours under the light microscope (Zeiss). The scratch areas were calculated using Wimasis.

2.6. Reverse transcription quantitative PCR assay

After 48 hours of 60 µM doxycycline treatment, the treated and untreated cells were collected. Total RNA was isolated via RiboExTM solution (Geneall) following the provider's instructions and total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific). smART First Strand cDNA Synthesis kit (EURX) was used to synthesize cDNA. The quantitative PCR reaction was performed on Applied Biosystem 7500 with a total volume of 20 µL. Primer sequences, including housekeeping *β-actin*, are shown in Table 1, and qPCR cycles are shown in Table 2. $2^{-\Delta\Delta Ct}$ method was used to show the relative quantification.

Table 1
Primer sequences.

Genes	Forward sequence (5′3′)	Reverse sequence (5′3′)
β-actin	CTCTGTGTGGATTGGTGGCT	GCAGCTCAGTAACAGTCCGC
Mmp9	GATCCCCAGAGCGTTACTCG	GTTGTGGAAACTCACACGCC
Bax	GAGGACTCCAGCCACAAAGA	CGAGCTGATCAGAACCATCA
Bcl-2	TATATGGCCCCAGCATGCGA	GGGCAGGTTTGTCGACCTCA
Caspase3	GGAGCTTGGAACGCGAAGAA	ACACAAGCCCATTTCAGGGT
Parp1	TCTACTTTGCTGATATGGTGTCC	TGGGTAACTTGCTGATGTGAG

Table 2
qPCR protocol.

Step	Cycles	Temperature	Time
Polymerase activation	1	95 °C	2 min
Denaturation		95 °C	5 s
Annealing	40	60 °C	10 s
Extension		72 °C	30 s

2.7. Statistical analysis

Comparisons between replicate groups were calculated with one-way ANOVA by GraphPad Prism 9.3.0. The significance of the results was set at $p < 0.05$.

3. Results and discussion

3.1. Doxycycline reduces the viability of rat glioma cells

The effects of different doxycycline dosages on cell viability after 48 hours were shown with MTT assay. As shown in Fig. 1A, doxycycline inhibited the proliferation ability of rat

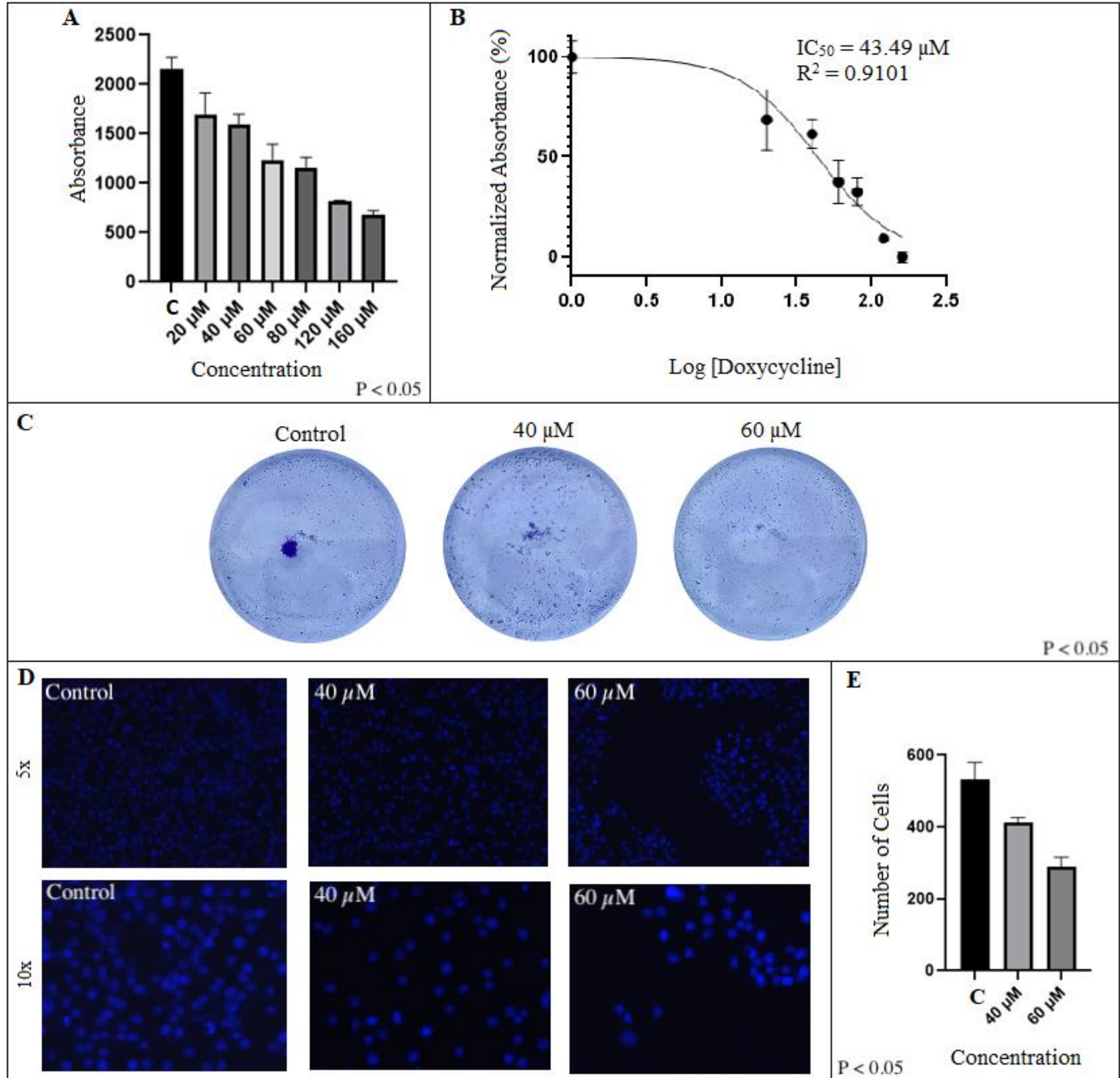

Fig. 1. Doxycycline inhibits cell viability and colony formation ability of rat glioma cells. (A) The viability of the cells after 48h doxycycline treatment decreased in a dosage dependant manner ($P < 0.05$). (B) IC_{50} was calculated as 43.49 µM via GraphPad Prism 9.3.0. (C) Doxycycline notably decreased the colony number in both groups ($P < 0.05$), 40 µM and 60 µM. (D-E) DAPI stained nuclei number was counted at 5x view and the number is significantly reduced in 60 µM doxycycline-treated cells ($P < 0.05$).

glioma cells in a dose-dependent manner. IC_{50} value was found as 43.49 µM (Fig. 1B), considering the viability values of the untreated group cells as 100%. In addition, the R^2 value, the proportion of the variance for a dependent variable, was calculated as 0.9101 (Fig. 1B).

3.2. Doxycycline affects the colony formation capability of rat glioma cells

To confirm the cytotoxicity of doxycycline on the cells, a clonogenic assay was applied. To determine the effects of doxycycline on cells progressively, the test was performed using 40 µM and 60 µM dosages to compare with the control cells. While the number of colonies in control cells is 583, 492 colonies in 40 µM and 419 colonies in 60 µM treated cells were counted, which shows the reduced number of colonies in treated rat glioma cells (15.6% decrease in 40 µM and 28.1% decrease in 60 µM treated cells) (Fig. 1C). Next, DAPI stained nuclei were counted and the number of nuclei reduced in 60 µM doxycycline-treated cells (Fig. 1D & 1E), 22.7% and 45.78% decrease were observed in 40 µM and 60 µM treated cells, respectively. The decrease in the number of cells in 40 µM treated cells was not found statistically significant.

3.3. Doxycycline inhibits the migration of rat glioma cells

Migration ability of rat glioma cells with doxycycline treatment was measured by wound-healing assay. The cells were scratched with a sterile pipette tip in the absence or presence of doxycycline. Gap areas measured after 24 hours, and 48 hours treatment and wound areas were wider of doxycycline-treated cells than in the control group (Fig. 2A). Results showed that 48-hour treatment with doxycycline reduces migration efficiency of the cells by 13,55% and 38,36% in 40 µM and 60 µM doxycycline-treated cells, respectively (Fig. 2B).

3.4. Doxycycline alters the expression of distinct apoptotic genes

Mmp9, a member of the matrix metalloproteinase gene family, is responsible for extracellular matrix degradation in mammals and increased gene expression level is observed in metastatic mammalian cancer cells (Morini et al., 2000). To determine the reduction effect of doxycycline on rat glioma

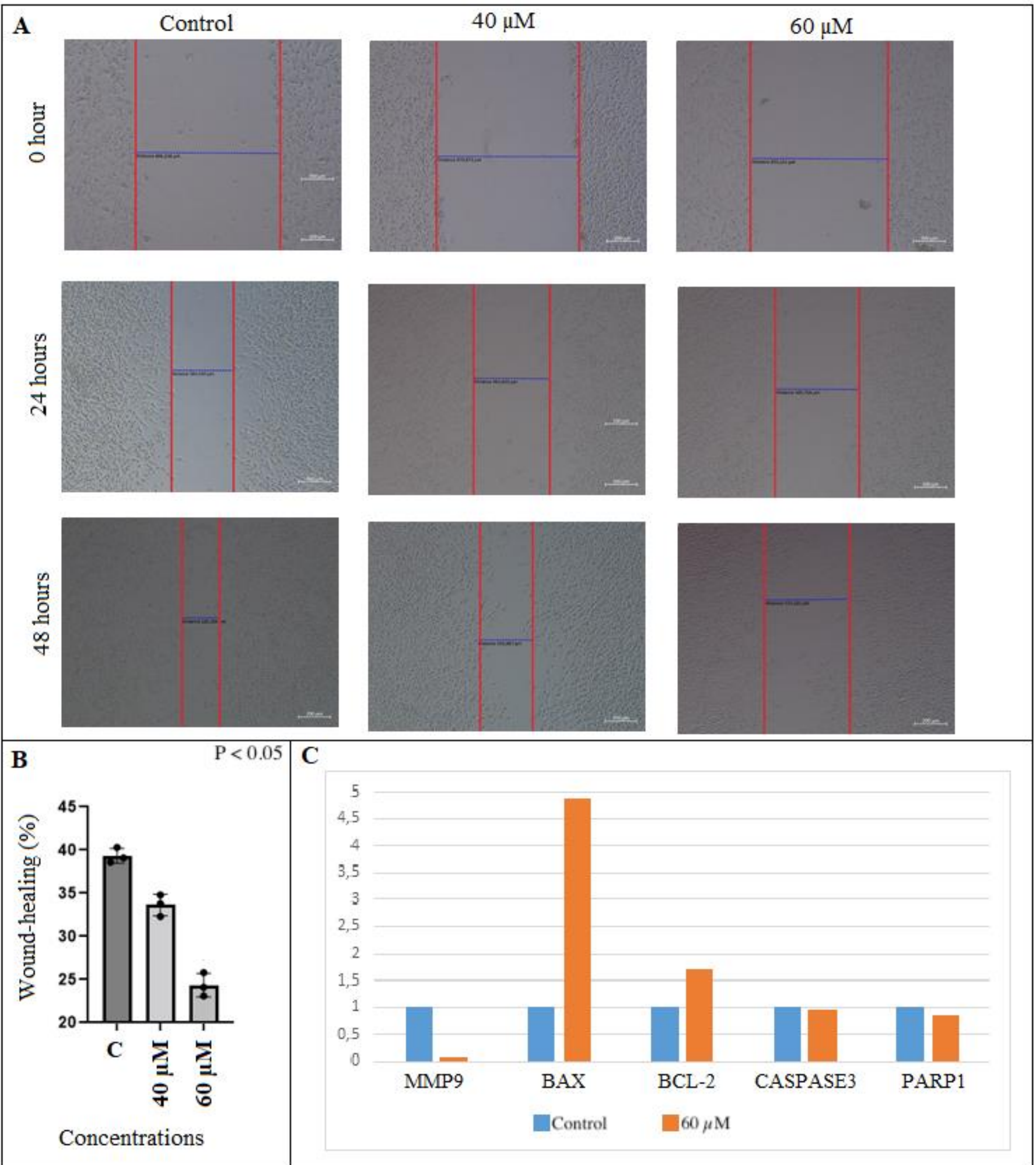

Fig. 2. Doxycycline inhibits migration ability and affects the expression of some apoptotic genes of rat glioma cells. (A) Scratch areas of 40 µM and 60 µM doxycycline-treated cells captured after 24h and 48h. (B) Migration ability was decreased by 13,55% and 38,36%, respectively ($P < 0.05$). (C) Relative quantification of *Mmp9*, *Bax*, *Bcl-2*, *Caspasex3*, and *Parp1* between control and 60 µM treated rat glioma cells.

cells, *Mmp9* expression in 60 µM doxycycline-treated cells for 48 hours was analysed. As demonstrated in Fig. 2C, doxycycline significantly inhibited the expression of *Mmp9* by decreasing it approximately 0.9-fold change. After 48 hours with 60 µM doxycycline treatment, doxycycline strongly induced the expression of *Bax* with approximately 3.5-fold change increase (Fig. 2C), which is a pro-apoptotic gene and has decreased level of gene expression in cancer cells (Miyashita et al., 1994). The expression of *Bcl-2*, which is an anti-apoptotic gene and ensures cell survival (Kelly and Strasser, 2020), was also increased 0.6-fold change in the presence of doxycycline (Fig. 2C). However, doxycycline did not have a significant effect of the expression of *Caspase3* (Fig. 2C), a pro-apoptotic gene (Alnemri et al., 1996), and *Parp1* (Fig. 2C) which is a proliferative gene and its increased gene expression levels detected in cancer cells (Pascal, 2018).

4. Conclusion

Various treatment approaches such as chemotherapy, radiotherapy, immunotherapy, and gene therapy are applied in cancer treatment and the mechanism of action of chemotherapy is primarily to stop the growth of cancer cells and then to kill the cells with a cytotoxic effect (Liu, 2009). However, while chemotherapy drugs can kill cancer cells, they also kill healthy cells where cancer cells form and metastasize, so the side effects of chemotherapeutic drugs can be dangerous for patients (Umfress et al., 2021). Since doxycycline has a bacteriostatic effect, its usage in cancer treatment might prevent the side effects of cancer treatments. Studies showed that doxycycline has high anti-metastatic activity and low cytotoxicity in cancer cells (Sun et al., 2009; Liu et al., 2015). Due to the lack of studies on glioma cells, rat glioma cells were used to investigate the

effects of doxycycline in this study.

In the current study, the cytotoxic effect was measured using MTT assay and IC_{50} value of rat glioma cells were determined as 43.49 µM for 48 hours in a dose-dependent manner. Qin et al. (2015) showed that IC_{50} values of doxycycline on NCI-H446 and A549 cell lines, which are lung cancer cells, were between 1 and 2 µM after 48 hours of application of doxycycline and it was stated that lung cancer is much more sensitive to doxycycline than other cancer cell types. In addition, Liu et al. (2021) demonstrated that for pancreatic cancer cells, Panc-1 and Aspc-1, IC_{50} values were found to be 987.5, 99.64, and 50.02 µM after 48, 72, and 96 hours of doxycycline administration, respectively. Doxycycline also reduces mitochondrial function and protects cells from death caused by hypoxia conditions in glioma cells LNT-229, G55, and U343 and it is effective at high concentrations (Luger et al., 2018). Likewise, anti-proliferative effects occur only at high dosages in U251HF, U87, and LN229 glioma cells and it may be because of GBM tumors being resistant to cytotoxic intervention compared to other solid tumours in clinical settings (Wang-Gillam et al., 2007). Since the dose and time-dependent effects of the determined active substance on different cells are expected in the scientific framework, differences in IC_{50} values in the literature are considered normal.

In this study, the colony formation test was performed on glioma cells with the determined doxycycline dosages and control group. As a result of the test, a significant dose-dependent decrease in colony number was shown. Similarly, Zhang et al. (2017) found that doxycycline significantly reduced colony formation of MCF7 and MDA-MB-468 cell lines, which are breast cancer cells, in a dose-dependent manner. Wound healing testing was performed to consolidate these results. A statistically significant decrease was observed in the wound healing rate depending on the dosage in this study. As Qin et al. (2015) show, doxycycline also decreases wound healing and migration ability of lung cancer cells after 24 and 48 hours. Cells were stained with DAPI after dosage applications for morphological examination. They were captured under microscope to determine the cell numbers and a considerable reduction was shown in the cell number of the 60 µM doxycycline-treated group. As the last step of the experiment, the effects in gene expression levels of the cancer related genes were measured by RT-qPCR compared with the control group. An important reduction in *Mmp* expression levels was determined in studies performed by applying doxycycline to cancer cells. Doxycycline degraded the extracellular matrix and basement membrane and inhibited *Mmp2* and *Mmp9* enzymes, which are involved in the cancer invasion steps, reducing lung cancer metastasis by inhibiting the degradation of the extracellular matrix and basement membrane. Similarly, in this study, a decrease in *Mmp9* expression was observed in the cells treated with 60 µM doxycycline with RT-qPCR test. Wang et al. (2016) showed that, in H446 cells, a small lung cancer line, doxycycline decreased anti-apoptotic gene *Bcl-2* expression and caused an increase in the pro-apoptotic genes *Bax* and *Caspase3* genes. In this study, it was observed that while the *Bax* expression levels increased, the *Bcl-2* level also increased. It is unexpected for genes that often work in reverse. However, the 350% increase in the *Bax* expression level compared to the 60% increase of *Bcl-2* expression level creates a difference in the level between them. Furthermore, the effect of doxycycline is to prevent the cell from proliferating by reducing its metabolism rather than driving it into apoptosis; therefore, an increase in *Bcl-2* level can be expected. Although there was a decrease in *Caspase3* and *Parp1* levels in this study, no significant decrease was observed, which supports the fact that doxycycline reduces cell proliferation instead of causing cell death. The mechanism of action of the active ingredient may vary depending on the cell line examined.

In conclusion, significant results were obtained particularly in cytotoxicity and proliferation tests. In the gene expression test with cancer-related genes, the significant result was observed in the *Mmp9* and *Bax* genes. Doxycycline has been found as a promising agent especially in the prevention of cancer metastasis, since it suppresses the *Mmp9* gene and it does not have a high cytotoxic effect, and reduces the migration ability of cells. In this study, the genes involved in apoptosis were examined, however more cancer-related genes need to be tested for more comprehensive studies.

Additionally, in order to determine whether post-transcriptional modifications have an effect on the mRNA products of the relevant genes, it should be analysed by western blot method, which determines the expression level at the protein level. RNAi and CRISPR/Cas methods can be used to determine the signaling pathways that doxycycline affects to be used in the treatment of glioma cancer, and its effects at the molecular level can be understood in more detail. Doxycycline is considered to have potential use in the treatment of glioma cancer, either alone or as a complementary therapy (Hassan et al., 2022), if promising results are obtained in sufficient quantities from *in vitro* and *in vivo* studies.

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Research article

Bioactive component analysis of seed coat hexane extract of Ardahan (Türkiye) walnut

Esra Palabiyik*[1], Handan Uguz[2], Bahri Avci[1], Ayse Nurseli Sulumer[1], Bilal Yilmaz[3], Hakan Askin[1]

[1] *Ataturk University, Faculty of Science, Department of Molecular Biology and Genetics, 25000, Erzurum, Türkiye*
[2] *Ataturk University, Faculty of Agriculture, Department of Field Crops, 25000, Erzurum, Türkiye*
[3] *Ataturk University, Faculty of Pharmacy, Department of Analytical Chemistry, 25000, Erzurum, Türkiye*

Abstract

The walnut seed coat (WSC), a significant waste product that is underappreciated compared to other walnut parts, is responsible for the emergence of biological potential, has a favorable and protective effect on health, and includes a wealth of bioactive phytochemicals. Considering this situation, the aim of the study was to clarify the bioactive components in the WSC hexane extract. A hexane WSC extract (H-WSC) was initially produced to conduct the analysis. Four chemical components in the walnut seed coat were identified using the gas chromatography-mass spectrometry (GC-MS) method. Accordingly, the main components in hexane extracts are β-sitosterol (%32.91) and ethyl iso-allocolate (%52.06); other components are 3-(octadecyloxy)propyl (9E)-9-octadecenoate (%8.41) and santa camphor (%4.45).

Keywords: *Gas chromatography-mass spectrometry; phytosterol; steroid; terpenoid; walnut seed coat*

1. Introduction

Since ancient times, people have used plants or items derived from them as supplemental sustenance, as well as for the prevention and treatment of chronic ailments. In terms of global gene diversity, Türkiye is regarded as one of the most significant regions (Karahan et al., 2020). The main causes of this predicament are its unique nature, geographic location, topography, and microclimate (Karakose, 2022). Despite there being about 11707 plant taxa, 3649 of them are endemic, which is a 31.82% percentage (Suzen and Atamov, 2022).

Juglans regia L., a member of the Juglandaceae family, is a type of walnut that is valued highly economically in agriculture. Although it is produced over a range that stretches from Central Asia to the Mediterranean, the majority of it is grown in temperate temperatures (FAO, 2024). In Türkiye, walnut production has spread throughout the Mediterranean, Marmara, Aegean, Anatolian, and Black Sea regions, among other places (Komaki et al., 2019). Walnut seed coat (WSC), which is the woody structure between the seed separated from the outer protective surface, has a very rich chemical content consisting of mostly unsaturated fatty acids (MUFA and PUFA), lipids, proteins, minerals, phytosterols, tocopherols, esters and phenolic compounds (juglone, syringic and ellagic acid) (Martínez et al., 2010; Geng et al., 2021; Yang et al., 2022).

Oxidative stress occurs due to the deterioration of the balance between the antioxidant defense system and free radicals (hydroxyl radicals (OH•), singlet oxygen ($^1O_2$), superoxide anion radicals (O_2•-), and non-free radical species such as hydrogen peroxide (H_2O_2) and causes serious health problems by causing tissue damage (Uguz et al., 2022). Additionally, internal and external factors such as UV and increased glucose levels also contribute to ROS production (Zhu et al., 2024). In addition to showing chelating properties against

* Corresponding author.
E-mail address: esraozdemir.tr@gmail.com (E. Palabiyik).
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metals that cause oxidative stress, polyphenols have important properties such as forming stable chemical complexes by donating hydrogen and neutralizing free radicals by inhibiting the enzymes involved in the formation of reactive oxygen species (ROS) (Novotny et al., 2017; Demir et al., 2019; Bayrak et al., 2022). It has been supported by many studies that this phytochemical profile, which is used very effectively in traditional medicine and also found in WSC, has antioxidant, antimicrobial, antidiabetic, antitumor (Mates et al., 2023), anti-apoptotic (Askin et al., 2022a), hepato-renal protective effects (Palabiyik et al., 2022; Askin et al., 2022b), anti-inflammatory enzyme inhibition (Palabiyik et al., 2023) and anti-hyperlipidemic (Palabiyik, 2022) properties. Therefore, it has been amply demonstrated that polyphenols control numerous systems that are beneficial to health.

To extract the most phenol from the dried walnut seed coat, the solvent and associated technique were modified for this study. Hexane was employed as the solvent in this case, and the GC-MS method was used to characterize the chemical components.

2. Materials and methods

2.1. Plant materials

The walnuts to be used for the analysis were obtained from the Posof (Türkiye) district of Ardahan in the autumn season between September and October (2022).

2.2. Preparation of walnut seed coat for analysis

After extracting the seed coat samples from the walnuts provided, they were dried in a thin layer at room temperature without being exposed to direct sun. All processes related to the plant were carried out in Atatürk University, Faculty of Science, Genetics Laboratory.

2.3. Hexane extraction of walnut seed coat

The previously dried WSC was weighed and powdered to a weight of 25 g. The solvent 500 ml of hexane (C_6H_{14}) was added, and the mixture was stirred magnetically for 72 hours. Filtering was performed to remove waste materials from the extracted plant sample in a reflux setup (4 hours, 60-80°C). Evaporator was used to remove the solvent remaining in the sample (155 rpm, 50°C). The sample was then dried in the oven and prepared for GC-MS analysis after this procedure, which was carried out three times to increase the yield (Khongthaw et al., 2023; Alkali et al., 2024).

2.4. GC-MS system and chromatographic conditions

GC-MS analyses were performed on an Agilent 7820A gas chromatography system with a 7673 series autosampler chemstation and a 5977 70 series mass selective detector. For separation, an HP-5 MS segment (30 m 0.25 mm I.D., USA) with a 0.25 µm film thickness was used. The input and transfer lines had temperatures of 250 and 300°C, respectively. Injection capability was determined as 1µl indivisible injection mode, carrier gas helium, flow rate 1ml/min, and ionization energy 70 eV (Mawlid et al., 2023; Rutkowska et al., 2023).

Different temperatures were applied while performing the GC-MS procedure. Accordingly, the adjustment was made to increase by 50°C for 1 minute, to increase by 20°C per minute for 1 minute at 100°C, to increase by 10°C per minute for 1 minute at 180°C, and to increase by 5°C per minute for 1 minute. The components of the extract's chromatograms and mass spectra were identified by contrasting them with the reference standard substance.

2.5. Identification of components

In the National Institute of Standards and Technology Library Version (2005), Software, Turbomass 5.2, the range of the obscure segment was compared with the range of the part stored. By comparing the direct Kovats maintenance list and mass spectra with those obtained from the MS library, the pieces could be separated. Utilizing the National Institute of Standards and Technology's database, which has more than 62,000 cases, an understanding of the mass range GC-MS was guided. Every component's relative rate measure was calculated by comparing its typical pinnacle region to the total areas. The test materials' component names, atomic weights, and structures were uncovered.

3. Results

The solvents used during extraction have different polarities, which allows the extracted substance to be separated into different compounds. Plants contain biocomponents that determine bioactivities (Alawode et al., 2021).

Table 1
Chemical composition of compounds identified in walnut seed coat plant.

Peak	Retention Time (min.)	% of total	Compound	Molecular Formula
1	29.52	32.91	β-sitosterol	$C_{29}H_{50}O$
2	33.41	8.41	3-(octadecyl-oxy) propyl (9E)-9-octadecenoate	$C_{39}H_{76}O_3$
3	34.36	52.06	Ethyl iso-allocholate	$C_{26}H_{44}O_5$
4	34.67	4.54	Santa Camphor	$C_{10}H_{16}O$

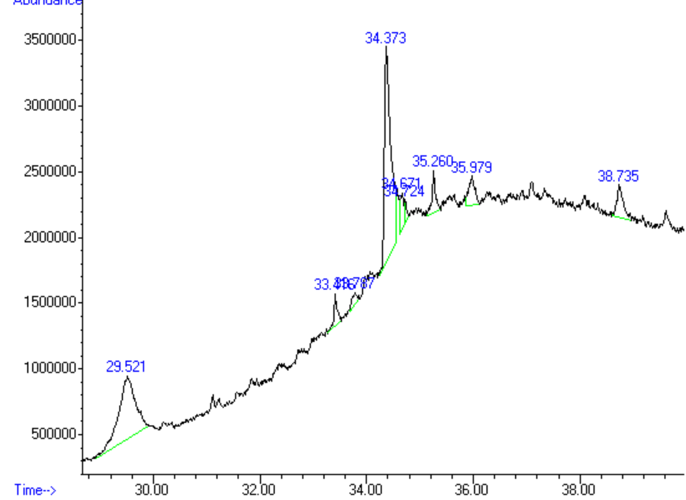

Fig. 1. GC-MS chromatogramı of H-WSC

Accordingly, hexane, a non-polar solvent, was used in our study and four components were detected in the extract of WSC. According to GC-MS analysis, Ethyl iso-alcholate (52.065%),

an ester, and β-sitosterol (32.91%), a steroid, were determined as the main components, while 3-(octadecyloxy) propyl (9E)-9-octadecenoate (8.407%) an unsaturated fatty acid, and the terpenoid Santacamphor (4.455%) were determined as other components. Table 1 provides a detailed summary of the bioactive substances identified in the walnut seed coat. In addition, the GC-MS chromatogram of these substances is presented in Fig. 1 and the three-dimensional view is presented in Fig. 2.

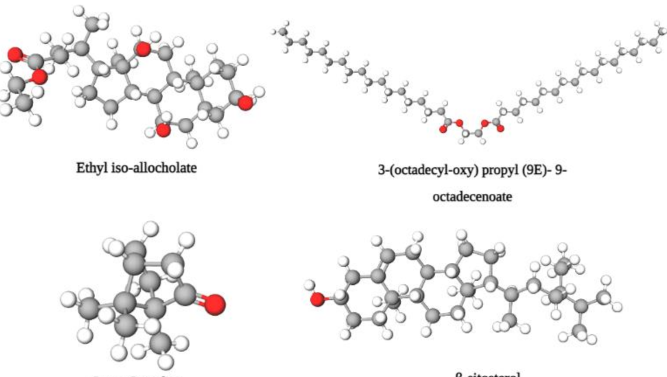

Fig. 2. 3D visualization of H-WSC's bioactive components (KingDraw).

4. Discussion

Considering the available literature, it is possible to say that the most important situation is the existence of therapeutic agents that have little or no side effects and are effective, natural, and easily available.

Table 2
Biological activities of chemical compounds reported in H-WSC.

Chemical constituents	Biological activities	References
β-sitosterol	Anti-inflammatory effect, Anticancer, Hepatoprotective activity, Antioxidant activity, Antidiabetic effect, Cardioprotective, Antimicrobial	von Holtz et al., 1998; Park et al., 2007; Paniagua-Pérez et al., 2008; Sujila et al., 2014; Nasution et al., 2015; Liu et al., 2019; Nweze et al., 2019; Devaraj et al., 2020; Lin et al., 2020
3-(octadecyl-oxy)propyl (9E)-9-octadecenoate	Antifungal	Rech et al., 1998; Diab et al., 2021; El-Naggar et al., 2023
Ethyl iso-allocholate	Anti-inflammatory effect, Antiviral, Antioxidant, Antibebesial potential, Anticancer, Antimicrobial	Okoye et al., 2011; Boligon et al., 2013; Malathi and Ramaiah, 2017; Prakash et al.,2019; Thakur and Ahirwar 2019; Johnson et al., 2020; Poochi et al., 2020; Guz et al., 2021; Shah et al., 2021; Arsana et al., 2022
Santa Camphor	Antifungal, Antibiofilm, Analgesic, Antimicrobial, Antiviral, Antitussive, Anti-inflammatory	Sokolova et al., 2017; Ivanov et al., 2021; Dos Santos et al., 2021

One of the key elements in our study, β-sitosterol (BS), is a steroid that belongs to a significant subclass of phytosterols that are typically found in plants, animals, sea creatures, and fungi. The hydrophobic linear and cyclic molecule BS, which is chemically similar to cholesterol, is considered safe (or non-toxic) because it does not have any negative side effects and works as a supplement. (Choi et al., 2017; Ravi et al., 2023; Mekarunothai et al., 2024). According to studies, BS may offer a wide range of advantages, including anti-inflammatory, anticancer, antibacterial, angiogenic, immunomodulatory, antioxidant properties (Rashed, 2020) and antidiabetic (by inhibition of α-Amylase enzyme) (Ravi et al., 2023), Additionally, studies on animals have revealed that it has no cytotoxic or genotoxic effects (Paniagua-Pérez et al., 2005). In addition, β-Sitosterol's lipophilic properties make it easier for it to be absorbed into the human body and can be considered as a nutritional supplement (Ravi et al., 2023). As a result, it has a significant application in the food and pharmaceutical industries (Rashed, 2020).

The microbial, anti-cancer, anti-fungal and diuretic anti-inflammatory properties of ethyl iso-allocholate, a steroidal molecule obtained in the H-WSC extract, have been reported in other studies (Gaanapriya et al., 2024; Kumar et al., 2024; Okasha et al., 2024). This substance, also known as ethyl cholate, slowed the growth of tumors and caused a decrease in liver metastasis and angiogenesis (Thakur and Ahirwar, 2019). It has demonstrated potent anticancer properties against A549 lung cancer cells *in vitro* and *in vivo* and by showing significant cytotoxicity to HepG2, hepatocellular cancer cells, through upregulation of PCNA, Bax, and caspase 3 activation (Pan et al., 2016; Fachriyah et al., 2019; Sampathkumar et al., 2020). *Bersama engleriana*, which also contains ethyl iso-allocholate, is used in the treatment of various stomach disorders (abdominal pain, colic, diarrhea, cholera, intestinal worms, amebiasis, and dysentery) (Sampathkumar et al., 2020). It was also supported by the study conducted in 2020 that the viral genome has an antiviral character, especially for SARS-CoV, by inhibiting the binding of the viral genome to target protein targets such as angiotensin-converting enzyme 2 (ACE2) and main protease (MPro) (Poochi et al., 2020).

Among other components, 3-(octadecyloxy)propyl (9E)-9-octadecenoate, a bioactive phytochemical compound, plays an important role in the preservation of fresh foods, as well as having an antifungal effect against human and plant fungal pathogens (Abubacker and Devi, 2014; Bhosale et al., 2021). Santa camphor, one of the most well-known bicyclic monoterpenoid compounds obtained naturally and synthetically from turpentine oil, has many remarkable features such as being affordable, having a robust molecular structure, and being synthetically convertible into useful derivatives. These properties of Santa camphor open a new avenue for different applications such as cosmetic products such as creams, lotions, and ointments, as well as fresheners and food products (Dardeer et al., 2022). It is also found in various cell lines (MRC-5, HT-26, HCT116) and has a cytotoxic effect (Nikolić et al., 2015). It is also considered an excellent antifungal by reducing the formation of biofilm and hyphae, that is, the virulence effect of the fungus (Ivanov et al., 2021).

4. Conclusion

It is crucial to find or develop new, powerful medication molecules that can treat diseases including cancer, diabetes,

immune-suppressing illnesses, bacterial and fungal infections, and cholesterol, which is a major factor in the development of many illnesses. Studies done recently with a lot of tenacity have revealed a progression in this direction. The usage of natural products (such as plant sources) since antiquity is also regarded as an amazing factor in the development of curative agents. In our research, the hexane extract of walnut seed coat was found to contain some important chemicals, including β-sitosterol, 3-(octadecyloxy)propyl (9E)-9-octadecenoate, ethyl isoallocholate and Santa campor, according to the results of GC-MS analysis. Accordingly, future studies may reveal that these identified substances are necessary agents for the medical field, and may also help in the evaluation of their pharmacological activities.

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Research article

Investigation of the effects of *Eremurus spectabilis* Bieb. lyophilized and nanoparticle extracts on the cellular and enzymatic immune system in experimentally-induced hepatocellular carcinogenesis in rats

Dilara Genc[1], Ismail Celik*[1]

[1] *Van Yuzuncu Yil University, Sciences Faculty, Department of Molecular Biology and Genetics, 65080, Van, Türkiye*

Abstract

Cancer is the leading cause of death after cardiovascular diseases. Hepatocellular carcinoma (HCC) constitutes the majority of primary malignancies of the liver. In this study, the effects of *Eremurus spectabilis* lyophilize and nanoparticle plant leaves extracts (LPLE-NPLE) were carried out on cellular and enzymatic immune system of hepatocellular carcinoma experimentally induced with diethylnitrosamine (DEN) in rats. The aims of study, it is to investigate the plant leaf extracts on T lymphocyte subsets mature T lymphocyte (CD3+), helper T lymphocyte (CD4+), suppressor-cytotoxic T lymphocyte (CD8+) and the CD4+/CD8+ as cellular immune systems. Further, it is aim to determinate activity of myeloperoxidase (MPO) and adenosine deaminase (ADA) activities in lung and spleen tissues of rats as enzymatic immune systems too. The study was conducted on six groups in each group 6 rats as normal control (NC), cancer control (CC), cancer+50 mg LPLE/kg (CLPLE1), cancer+100 mg LPLE/kg (CLPLE2), cancer+50 mg NPLE/kg (CNPLE1) and cancer+100 mg NPLE/kg (CNPLE2). To reveal the effects of the plant extracts in rats treated with two doses on cellular and enzymatic constituents of immune systems, the blood, spleen and lung samples were taken from rats at the end experiment. CD3+, CD4+, CD8+ cells and CD4+/CD8+ ratio was analysed by flow cytometry in blood samples. Furthermore, MPO and ADA enzyme activities were analyzed in supernatants of the lung and spleen tissues. According to the obtained results; $CD3^+$ and $CD8^+$ T cells of CLPLE1 bases were statistically reduced compare with NC and CC groups. Again, a significant decrease was found statistically the CNPLE2 group compared to the NC and CC groups. $CD4^+$ T cells were significantly decreased compared to NC. On the other hand, ADA enzyme, which is an enzyme of the immune system, decreased in CC compared to NC, while it increased in lung and spleen tissues in CNPLE1, CNPLE2, CLPLE1 and CLPLE2 groups. Regarding the MPO; In the groups supplemented with plant extract, MPO enzyme activity increased in both lung and spleen tissues compared to NC and CC. According to these results, the manuscript results present some new data and original theory about healing effects of *E. spectabilis* LPLE and NPLE on experimentally induced cancer complications as constituent of immune system with DEN. But it was concluded that more studies are needed to reveal the mechanism of action of *E. spectabilis* in cancer treatment and its therapeutic use.

Keywords: *Cellular and enzymatic immune system; Eremurus spectabilis; hepatocellular carcinoma*

1. Introduction

In recent years, medical and economic problems caused by the occurrence of serious side effects from synthetic drugs have led to the idea that natural is always effective and side-effect free. Add to this the threat posed by many chronic diseases for which no cure is yet possible, and these concerns have become considerable. Plant-derived compounds are very important due to their various biological effects such as anti-obesity, anti-hypertension, anti-cancer, anti-inflammatory, anti-diabetic, antimicrobial, antioxidant and anti-Alzheimer (Sharifi-Rad et al., 2018; Salehi et al., 2019; Islam et al., 2020; Gulcin, 2020).

* Corresponding author.
E-mail address: icelik@yyu.edu.tr (I. Celik).
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In addition, ecological approaches resulting from environmental pollution in industrialized countries reinforce this idea. Due to many factors, herbal treatment has become popular again. Cancer herbal treatment, also known as phytotherapy, is used as a complementary therapy alongside or after medical treatment, especially to reduce the side effects of medical treatment, correct and support the damaged immune system, increase the benefit of treatment, reduce the ability of cancer cells to metastasize, and kill cancer stem cells (Choudhari et al., 2020).

Globally, hepatocellular carcinoma (HCC) ratio is one of the most common types of cancer and is the second leading cause of cancer-related deaths. (Bray et al., 2018). Notably, 83% of liver cancer cases are concentrated in underdeveloped regions, particularly in Asia and Africa (Ferlay et al., 2015). The majority of HCC patients have a medical history involving hepatitis B virus (HBV), chronic liver disease or cirrhosis hepatitis C virus (HCV). The incidence of liver cancer varies depending on the underlying cause of cirrhosis, with hepatitis B infection being the primary cause in our country. Furthermore, cirrhosis and hepatitis C can also result from alcohol consumption (El-Serag and Rudolph, 2007).

Wild perennial herbaceous *Eremurus spectabilis* belongs to the *Eremurus* genus within the Liliaceae family. Asphodel, an extinct wild plant species, once served as both a nutritious vegetable and an ornamental soil plant in various industries. Approximately 50 species comprise this genus, predominantly found in southern and Asian regions, including the Middle East, the Caucasus, and Turkey (Tuzlaci, 1985). Notably, *E. spectabilis* and *E. cappadocus* have been documented in Anatolia (Gungor, 2002). *E. spectabilis* has recently gained popularity as an ornamental geophyte plant, particularly in mild climate zones, and is extensively used in cut-flower production practices (Schiappacasse et al., 2013).

The shoots and leaves of these plants are commonly consumed as a vegetable and are valued for their medicinal properties. In traditional medicine, various parts of the plant are utilized to address ailments such as fungal diseases, diabetes, jaundice, and liver disorders (Baytop, 1984; Tuzlaci and Dogan, 2010). Also, traditionally, the leaves of *E. spectabilis* are used for gastrointestinal disorders. In a study conducted on rats experimentally induced with indomethacin, stomach ulcers were formed, and as a result of the analyzes performed at the end of the study, it was determined that *E. spectabilis* had an antiulcer effect (Karaoglan et al., 2018). On the other hands, plants, which are also very rich in minerals, are good sources of vitamin C and antioxidants. It had been found that *E. spectabilis* is rich such as K, Ca, Na P, Mg, Fe, Zn, Cu mineral constituent and also very rich in vitamin C. (Tuzlaci, 1987).

In during years, there has been a rising interest in examining on novel hepatoprotective drugs derived from natural sources, offering promising avenues for therapeutic advancement. Among these, *E. spectabilis* stands out as a particularly promising medicinal plant due to its antioxidant properties and pharmaceutical potential. However, despite its potential benefits, there has been a notable absence of experimental studies investigating the medicinal effects of this plant on hepatocellular carcinoma and its immunological impacts in rats. The aims of study to assess the therapeutic effects of different extracts from the plant's leaves on immune cellular biomarkers including CD3+, CD4+, CD8+, and the CD4+/CD8+ ratio, using flow cytometry analysis of blood samples. Additionally, the enzymatic immune system, specifically myeloperoxidase (MPO) and adenosine deaminase (ADA) enzyme activities will be evaluated in lung and spleen tissues during the experimental induction of hepatocellular carcinogenesis in rats using diethylnitrosamine.

2. Materials and methods

2.1. Chemicals

The chemicals used in the study were supplied from Sigma Chemical Co. The kits for cellular elements of the immune system supplied from Elabscience Ltd.

2.2. Plant materials

The plant material was collected in Çınardı (Pizongan) village of Korkut district of Muş on 22.04.2021. The plant authenticity was confirmed by Prof. Dr. Fevzi ÖZGÖKÇE from the Department of Molecular Biology and Genetics at Van Yuzuncu Yil University. The herbarium number of *E. spectabilis* was determined to be 16270 and a sample of the plant was stored at the Van Yuzuncu Yil University Van Herbarium (VANF).

2.3. Preparation of plant extracts

In the preparation of alcohol extracts, the alcohol concentration used affects the biochemical profile of the extract (Vizzoto et al., 2018). They prepared *E. spectabilis* extracts with different alcohol concentrations and found that the antioxidant influence of the different extracts was higher when 80:20 (%) ethyl alcohol: water was used compared to 70% ethanol and pure ethanol. Therefore, in the study, ethanol extracts were prepared using 80% ethanol. 100 g of *E. spectabilis* leaves were blended with 500 ml of 80% ethanol solution and homogenized. The samples were extracted overnight in a magnetic stirrer at room temperature, surrounded by foil. Afterwards, the extracts were first filtered with a coarse strainer and the coarse pulp was removed. The resulting filtrate was pipetted into tubes and centrifuged at 3500 rpm for 5 minutes. Samples removed from the centrifuge were passed through coarse filter paper and collected in a screw-cap bottle protected from light. The filtrate solvents were firstly removed by the evaporation at 60 atm pressure at 37°C. Then, the pressure of the device was lowered to 18 atm and the water was removed to obtain a concentrated extract. The concentrate plant extract has divided to the tubes as 15 ml. Tubes were stored at -80°C overnight for nanoparticles and lyophilized process.

2.4. Preparation of the lyophilized plant extract

Water and ethanol extract was prepared using a revised method based on Dalar and Konczak (2013) approach. Following the extraction process, the concentrated extract sample was frozen at -50°C and subjected to lyophilization under 50 millitorrs pressure conditions for 7 days.

2.5. Characterization of plant extract and synthesis of nanoparticle

After the plant extracts were centrifuged and filtered, the density of the supernatant obtained was doubled with a rotary evaporator. 10 ml of the concentrated extract was taken and

placed in the vial. While this vial was mixed in the magnetic stirrer at 5000 rpm, ammonium persulfate $(NH4)_2S_2O_8$ as an initiator, glutaraldehyde solution $[OHC(CH_2)_3CHO]$ as a cross-linker and as an accelerator N,N,N',N'-Tetramethyl ethylenediamine $[(CH_3)_2NCH_2CH_2N(CH_3)_2]$ were added respectively. The mixture was centrifuged at 10000 rpm for 4 hours. It was accepted that nanoparticles were synthesized when there was a color change in the mixture. After centrifugation, the remaining filtrate was dried (Sahiner and Sengel, 2016).

2.6. Animals and experimental design

Wistar albino rats were employed in this research. Thirty-six Wistar albino rats, aged between 3 and 4 months and weighing an average of 181-258 g, were procured from the Van Yuzuncu Yil University Experimental Animal Research Center. The study was conducted in accordance with the approval obtained from the Van Yuzuncu Yil University Experimental Animals Unit Ethics Committee on September 29, 2022, under the reference number 2022/09-06. The rats were housed at a temperature of 22 ± 2°C with a 12-hour light/12-hour dark cycle and provided ad libitum access to food and water.

For this investigation, the 36 rats were divided into six groups, each comprising six rats, one of which served as a control. The study duration spanned 10 weeks. The grouping of the rats for the study was as follows:

2.6.1. Normal control (NC)

Rats in this group, which would not be exposed to any treatment, were fed with standard rat chow and tap water as ad libitum.

2.6.2. Cancer control (CC)

Rats in this group were received to 150 mg EN/kg body weight (bw) once a month and 200 mg Tiyoasetamid (TAA)/kg bw once a week as intraperitoneal (ip) (Hassan et al., 2017).

2.6.3. Cancer+50 mg/kg lyophilized plant extract (CLPE1)

Rats in this group were received to 150 mg EN/kg bw once a month and 200 mg TAA/kg bw once a week as ip. Additionally, the rats received 50 mg CLPE/kg bw per day orally by gavage.

2.6.4. Cancer+100 mg/kg lyophilized plant extract (CLPE2)

Rats in this group were received to 150 mg EN/kg bw once a month and 200 mg TAA/kg body weight once a week as ip. Additionally, the rats received 100 mg CLPE/kg bw per day orally by gavage.

2.6.5. Cancer+50 mg/kg nanoparticle plant extract (CNPE1)

Rats in this group were received to 150 mg EN/kg bw once a month and 200 mg TAA/kg body weight once a week as intraperitoneal (ip). Additionally, the rats received 50 mg CNPE/kg bw per day orally by gavage.

2.6.6. Cancer+100 mg/kg nanoparticle plant extract (CNPE2)

Rats in this group were received to 150 mg EN/kg bw once a month and 200 mg TAA/kg body weight once a week as intraperitoneal (ip). Additionally, the rats received 100 mg CNPE/kg bw per day orally by gavage.

2.7. Preparation of tissues supernatant

At the end of the 10 weeks, the experimental phase was finalized by sacrificing the rats and taken the tissues such as spleen, lung and blood, samples. To achieve this, the rats were anesthetized with ketamine (5 mg/100 g body weight) as intraperitoneal. Intracardiac blood samples using injectors were taken for the analysis of CD3+, CD4+, CD8+ cells, and the CD4+/CD8+ ratio via flow cytometry.

Following the dissection of spleen and lung tissues, the samples were transferred to petri dishes and washed with 0.9% NaCl solution before being preserved at -78°C until analysis. Subsequently, the tissues were homogenized for 5 minutes using a stainless-steel probe homogenizer (20 KHz frequency ultrasonic, Jencons Scientific Co.) after the addition of 50 mM ice-cold KH2PO4 (1:5 w/v) solution. The resulting mixture was then centrifuged at 10,000xg for 30 minutes at 4°C. The supernatants obtained were utilized for assessing MPO and ADA activities (Celik et al., 2009).

2.8. Biochemical analysis

ADA activity determination was made according to Bergmeyer. According to this method, ADA catalyzes the formation of deoxyinosine from adenosine. The ammonia released at this time, together with sodium hypochlorite and phenol/nitroprusside, forms dark blue indophenol in alkaline solution. The resulting dark blue indophenol was measured colorimetrically at 630 nm. (Bergmeyer, 1974).

MPO activity determination was performed according to the method described by Bradley et al. This method is based on measuring the absorbance at 460 nm of the product formed because of the reduction of H_2O_2 oxidized by MPO to O-dianisidine. (Bradley et al., 1982).

2.9. Flow cytometric examination

Blood samples for flow cytometric analysis of blood samples taken into standard blood count tubes with K3 EDTA and it was first passed through a Coulter Immunoprep Leucocyte Preparation System (Q prep) device. Monoclonal test for lymphocyte subgroups was added to 100 µl of the cell preparation passed through the Q prep device.

20 µl of antibodies (Immunotech Kid No: 8546859/ISOTON) were added and incubated for 15 minutes in the dark at room temperature. Monoclonal antibodies were then conjugated with ECD (Extracellular Domain) and PE (phycoerythrin). Among the conjugated monoclonal antibodies, CD3-ECD is specific for circulating mature T lymphocytes, and CD4-ECD/CD8-PE is specific for helper and cytotoxic T cells. Cells labeled with antibodies were analyzed using a Coulter Epics XL II flow cytometer.

2.10. Statistical analysis

The statistical analysis was conducted using the Minitab 14 software package for Windows, and all data were presented as mean ± standard deviation (SD). To assess differences between the means of the experimental groups, paired t-tests were

Table 1
Effects of the lyophilized and nanoparticle plant extracts on T Lymphocyte subsets (X̄±SD).

Groups	T Lymphocyte subsets.			
	CD3⁺	CD4⁺	CD8⁺	CD4⁺/CD8⁺
NC	81.57±8.99	35.79±6.95	17.97±3.53	2.05±0.58
CC	80.29±6.53	34.59±5.69	17.34±5.62	2.21±0.88
CNPLE1	74.03±9.33	28.7±7.48[b]	13.25±3.80	2.16±0.55
CNPLE2	61.79±11.17[a,b]	27.67±6.09[a,b]	12.9±6.24[a,b]	2.58±1.23
CLPLE1	63.77±6.31[a,b]	35.19±3.40	13.34±2.64[a,b]	2.60±0.50
CLPLE2	78.03±8.57	37.31±5.60	16.76±5.82	2.50±0.93

a: There is a significant difference compared to the NC group (p≤0.05).
b: There is a significant difference compared to the CC group (p≤0.05).

employed, with a significance level set at p ≤ 0.05.

3. Results and discussion

Flavonoids and polyphenolic molecules in plants have been studied for their various effective biological activities. However, although these plant molecules are used for different purposes, relatively little is known about their healing properties.

Table 2
Effects of the lyophilized and nanoparticle plant extracts on MPO activities of some tissues (X̄±SD).

Groups	Tissues	
	Spleen (U/g)	Lung (U/g)
NC	1443.1±237	1190.3±290.4
CC	1570.5±229.2[a]	1442.8±218
CNPLE1	1804±334	1374.1±118[a]
CNPLE2	1854.3±255[b]	1481±308[a]
CLPLE1	1872.7±137.5[b]	1763.3±153.7[b]
CLPLE2	1820±361	1348.2±237.4[a]

a: There is a significant difference compared to the NC group (p≤0.05).
b: There is a significant difference compared to the CC group (p≤0.05).

Table 3
Effects of the lyophilized and nanoparticle plant extracts on ADA activities of some tissues (X̄±SD).

Groups	Tissues	
	Spleen (U/g)	Lung (U/g)
NC	3.80±0.48	4.3±1.7
CC	1.75±0.46	1.01±0.47[a]
CNPLE1	5.80±1.60[b]	6.67±1.8[a]
CNPLE2	7.30±1.90[b]	7.0±1.8[a]
CLPLE1	3.30±0.60[a]	3.70±8.0
CLPLE2	4.05±1.01	4.04±2.0

a: There is a significant difference compared to the NC group (p≤0.05).
b: There is a significant difference compared to the CC group (p≤0.05).

This study designed to investigate the potential protective effects of *E. spectabilis* lyophilized and nanoparticle plant extracts on cellular and enzymatic immune system against in experimental Diethylnitrosamine-induced hepatocellular carcinogenesis in rats. It is very important to scientifically reveal the therapeutic properties of these plants, especially in various diseases. In recent scientific studies, it is known that *E. spectabilis* extracts have antioxidant, gastro protective, cytotoxic, antifungal and apoptotic effects. Asphodel has been found to be an important source of reducing power, free radical scavenging activity, phenolic substance content and metal chelating activity. It has been found that the abundance of vitamin C in asphodel is very important for immunity and that it quickly heals eczema, acne and urinary tract infection.

Diethylnitrosamine (DEN) is a compound used for tumor formation in experimental animals (Tolba et al., 2015). The dose applied, the age and gender of the rats are very important to cause liver cancer after DEN administration (Heindryckx et al., 2009). The reason why mice were selected at a younger age is that liver cancer occurs more rapidly because of rapid division of liver cells (Vesselinovitch and Mihailovich, 1983; Vesselinovitch et al., 1984; Hassan et al., 2017; Romualdo et al., 2017). Also, the reasons for choosing rats as experimental animals in liver cancer studies are their genetic similarities with humans, their short lifespan and reproductive characteristics (Tolba et al., 2015).

Many studies are carried out to follow the causes of cancer and the results of treatment methods. Quite long periods of time are required for experimental studies in cancer research. For example; While Karakurt (2018) was conducted for 21 weeks, Vuran (2021) was conducted for 24 weeks. Also, Moreira et al. (2015) carried out over a 10-week period. In this study, the experimental treatment period of the study lasted 10 weeks, considering the duration of studies in the literature. We think this is a reasonable period for experimental treatment.

According to the obtained results, CD3+ and CD8+ T cells of CLPLE1 bases were statistically reduced compare with NC and CC groups. In addition, a significant decrease was found in the CNPLE2 group compared to the NC and CC groups. While CD4+ T cells of CNPLE1 and CNPLE2 groups were significantly decreased compared to CC, CNPLE2 decreased compared to only NC (Table 1). Regarding with the MPO; in the groups supplemented with plant extracts, MPO enzyme activity increased in both lung and spleen tissues compared to NC and CC (Table 2). On the other hand, ADA enzyme, which is an enzymatic biomarker of the immune system, decreased in CC compared to NC, while it increased in lung and spleen tissues in CNPLE1, CNPLE2, CLPLE1 and CLPLE2 groups (Table 3).

Cytotoxic T lymphocytes consistently exhibit surface antigens and play a pivotal role in the anti-tumor immune response. Recent studies have highlighted a reduction in CD3+, CD4+, and CD8+ TIL (Tumor Infiltrating Lymphocytes) expression within HCC tumor tissues. Yarchoan et al. (2017) conducted immunohistochemistry staining on 29 HCC cases, revealing significantly lower T cell expression within tumor tissue compared to background liver tissue, with even lower expression observed on the tumor side than the non-tumor side. Moreover, the central tumor zone exhibited lower T lymphocyte expression and a smaller area of infiltration compared to the border zone (Zhao et al., 2019).

There are many studies examining ADA enzyme activity in cancerous tissues and cells. During of these studies, quite different results were obtained. Some researchers have concluded that cancerous cells increase ADA enzyme activity

(Durak et al., 1993; Koizumi et al., 1993). Some studies have suggested that ADA activity increases in direct proportion to the cancer stage. Sufrin et al. (1978) found that the increase seen in patients with bladder cancer was directly proportional to the stage of the cancer. They also observed a decrease in the ADA enzyme activity level of lung cancer patients after surgery. In another study, it was found that the ADA enzyme activities of patients given radiotherapy decreased significantly (Hiromu et al., 1970). Furthermore, the study performed by Uchida et al. (2017) where they investigated paracetamol toxicity on mice, an increase in MPO level was observed in the toxic groups. The study concluded that the increase in liver MPO is related to the damage occurring in the liver. In a toxicity experiment involving the use of fig seed as a treatment group, it was observed that MPO levels increased in the toxicity group but decreased in the group treated with fig seed oil, suggesting a potential reduction in neutrophil migration (Uchida et al., 2017).

Previous studies have proposed that the inhibitory effects on neutrophils, ADA, and MPO are associated with the suppression of cell activation. This is because MPO is abundantly expressed in neutrophils and directly influences their phagocytic activity (Nussbaum et al., 2013). Furthermore, it has been suggested that an increase in ADA may be related to immune system activation, implying a correlation between changes in immunity and ADA activity (Ozok and Celik, 2019). However, MPO is primarily linked to inflammation and neutrophils, serving as a marker of infiltration (Ozkol et al., 2012). According to these theories, during stimulation of the cellular immune system, other substances disrupting tissue and MPO are released from cells (e.g., reactive oxygen species and cytotoxic proteins) into the extracellular space.

The study results indicate an increase in ADA and MPO levels in HCC groups, consistent with findings in the literature. This may be attributed to carcinoma complications, with the elevation of liver MPO levels being closely associated with liver damage. Additionally, ADA activity is crucial in stimulating receptors that regulate extracellular adenosine concentrations, thereby modulating the inflammatory response (Antonioli et al., 2012). Furthermore, it is impossible to compare the results obtained in this study and generalize about the changes, since there is no study that shows changes in ADA enzyme activity in rats with experimental HCC in this model. However, such changes in ADA and MPO activity across cancer types may also provide good information about the course of treatments.

4. Conclusion

The observations made at the end of our research led us to summarize that the induced experimental hepatocellular carcinoma and the plant extract administration not only leads to decrease in cellular T lymphocyte subsets and causes generally an increase in the immune defense systems enzymes as MPO and ADA. Therefore, based on these results, it cannot be said with certainty that plant extracts contribute to cellular and enzymatic immune system elements in experimental HCC. Nevertheless, the results suggest that regular consumption of this functional food may prove beneficial in preventing chronic degenerative diseases. Furthermore, it was determined that further research is necessary to elucidate the mechanism by which *E. spectabilis* operates in cancer treatment and its potential therapeutic applications.

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Research article

In-vitro bioaccessibility and mineral content of two *Ribes* species growing in Cumalikizik village, Bursa Türkiye

Asuman Cansev*[1], Muge Kesici[2], Yasemin Sahan[3], Guler Celik[4], Aysegul Akpinar[5], Meryem Ipek[1]

[1] *Bursa Uludag University, Faculty of Agriculture, Department of Horticulture, 16059, Bursa, Türkiye*
[2] *Bahçeşehir University, Faculty of Engineering and Natural Sciences, Department of Molecular Biology and Genetics, 34353, Istanbul, Türkiye*
[3] *Bursa Uludag University, Faculty of Agriculture, Department of Food Engineering, 16059, Bursa, Türkiye*
[4] *The Scientific and Technological Research Council of Türkiye (TUBITAK), Bursa Test and Analysis Laboratory, 16190, Bursa, Türkiye*
[5] *Bursa Uludag University, Vocational School of Technical Sciences, Department of Park and Horticulture, 16059, Bursa, Türkiye*

Abstract

The fruits of the genus *Ribes*, also known as currant or gooseberries, can be consumed both as processed and fresh. These berries' health benefits have been well described in general but their biophysicochemical properties largely depend on geographical changes and genotype differences. Six *Ribes* genotypes including *Ribes rubrum* (RR1-RR4) and *Ribes nigrum* (RN1 and RN2) from Cumalikizik, Bursa were compared for their fruit properties, mineral content, and their bioaccessibility. Fruit characteristics were evaluated by analyzing fruit and seed number, soluble solids content (°Brix), fruit color properties, and pH. Potassium (K), calcium (Ca), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), and magnesium (Mg) contents of fruits were determined using ICP-OES (inductively coupled plasma optical emission spectrometry). The results indicated that all *Ribes* genotypes were rich in K, Ca, and Mg content while they had relatively poor in Mn, Cu, and Zn content. Bioaccessibility of K, Ca, Mg, Fe, Mn, Cu, or Zn was 85%, 84%, 63%, 30%, 50%, 37% or 44% respectively for two *Ribes* species. Significant differences were found between *R. rubrum* and *R. nigrum* genotypes in terms of fruit size and weight, bunch length, seed number, total soluble solids, pH and color, as well as Mn content and Zn accessibility. These data provide valuable information regarding the physicochemical properties, mineral content, and bioaccessibility of two currant species for breeding studies and show that the *Ribes* species is a good source of K, due both to its high content and considerable bioaccessibility. Further research should consider investigating the contents and bioaccessibilities of other nutritional factors that *Ribes* genotypes contain.

Keywords: *Bioaccessibility; mineral content; Ribes rubrum; Ribes nigrum*

1. Introduction

The genus *Ribes* (currants), which includes more than 150 species and varieties, is a member of *Grossulariaceae* family and is commonly grown in mild parts of the Northern Hemisphere (Soloshenko, 2018). Despite the confusion concerning *Ribes* taxonomy, today it is acknowledged that the genus *Ribes* has five subgenera: *Berisia* (European dioecious plants), *Grossularioides* (thorny currants), *Parilla* (South American natives), *Ribes* (currants) and *Grossularia* (gooseberries). The commercially cultivated forms of red and white currants belong to section-*Ribes* (or *Ribesia*) of subgenera

* Corresponding author.
E-mail address: auslu@uludag.edu.tr (A. Cansev).
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Ribes (Messinger et al., 1999; Hummer and Dale, 2010). In Türkiye, 8 species of the genus *Ribes* L. are grown, and many other species are observed in the natural flora. Although a few numbers of studies have been implemented on naturally distributed *Ribes* taxa in our country, it is worth noting that this number has increased in recent years (Celenk, 2015).

Ribes exist in high number of bioactive metabolites and flavor compounds, such as sugars and organic acids. These berries are also valued for their food and flavoring qualities, as well as the potential health benefits for their high content of phenolic compounds (Adina et al., 2017; Tian et al., 2019; Montiel-Sánchez et al., 2021). Currants are also well-known fruits for their healing properties and are frequently utilized in the food and pharmaceutical industries. Berry consumption has been presented to have benefits for the treatment of age-related neurodegenerative diseases, metabolic syndrome, and several types of human cancers (da Silva Pinto et al., 2010). Moreover, fruit and bunch properties, which are genotype-specific traits, are important quality attributes for currants. As one of the quality parameters, coloration has a significant effect on the customers that makes it distinguishable as well as the aroma and the taste (Mikulic-Petkovsek et al., 2016). Additionally, berries like currants display diverse colors depending on genotypes and this is an attractive characteristic for the customer choices since they associate better taste with color. Environmental conditions such as temperature, strongly influence fruit color and fruit development by influencing the accumulation of healthy functional phytochemicals (Wang and Zhang, 2001; Krüger et al., 2011; Liang et al., 2023).

Black currants (*R. nigrum* L.) and red currants (*R. rubrum* L.) are two commonly cultivated species of the genus *Ribes* worldwide. In 2022, the total global production of currants was 764498.98 tons, with two countries dominating the market. The Russian Federation was the leading producer with 509500 tons, followed by Poland with 145800 tons (FAOSTAT, 2024). *R. nigrum* is recognized as a good source of polyphenols, anthocyanin, phenolic acid derivatives, flavonols, and proanthocyanidins (Paunović et al., 2017; Staszowska-Karkut & Materska, 2020; Ejaz et al., 2023; Maria-Beatrice et al., 2023). *R. rubrum* is also another currant species that is highly valued for its nutrients and antioxidant components, and they're also known to be especially rich in potassium, calcium, and magnesium (Cvetković et al., 2021; 2022; González et al., 2022). Studies reported that fruits especially currants contain high levels of minerals that can differ according to genotype, cultural practices, soil, and environmental conditions (Paunović et al., 2017; Tian et al., 2023). In addition, although the mineral contents of freshly consumed currants have been well-documented, little is known about the bioaccessibility of the minerals. This is a crucial information because bioaccessibility is not directly proportional to content and bioaccessibility of a given compound may be low despite a high content in the plant. The portion of a phytochemical that is released from the food matrix in a form that is easily absorbed is known as its bioaccessibility (Hu et al., 2023). Numerous elements influence it, such as the nature of phytochemicals, the composition of food matrix, and the working of gastrointestinal tract (Jo et al., 2021; Domínguez-Fernández et al., 2022). The portion of a phytochemical that enters the bloodstream and is used by tissues and organs is known as its bioavailability (Dima et al., 2021). Usually, the concentration of phytochemicals and their metabolites in the bloodstream following intake is used to make this determination. Therefore, research on the bioaccessibility of plants, along with their chemical characterization, is of great importance in scientific research. Cumalikizik village in Bursa was declared a protected area in 1980; the legal protection has preserved the authentic village with its street pattern, wooden houses, cottages, and green areas. In particular, agricultural activities continue in and around the Cumalikizik (Ahunbay et al., 2014). Knowledge about fruit species growing in Cumalikizik village is limited to a few articles (Cansev et al., 2022). Therefore, the present study targeted to investigate the mineral contents (K, Ca, Mg, Fe, Cu, Zn, and Mn) and bioaccessibility, as well as fruit quality parameters (color, fruit and seed number, soluble solids content (°Brix) and pH) of the total six genotypes belonging to *R. nigrum* and *R. rubrum*, which are grown in Cumalikizik village, Bursa, with considerable economic interest.

2. Materials and methods

2.1. Sampling location

The present study includes six *Ribes* genotypes from *R. nigrum* and *R. rubrum* collected from Cumalikizik village in Bursa, Türkiye (Fig. 1; Latitude: 40° 11' 25.1340", Longitude: 29° 10' 20.1360"). The sampling area is located at an altitude of 360 meters with surrounding natural chestnut and pine forests, and the soil is rich in organic matter with approximately 50% loamy structure and an average pH of 6.5.

Fruits of a total 6 *Ribes* genotypes (4 *R. rubrum* and 2 *R. nigrum*; abbreviated as RR1-RR4 and RN1 and RN2 from here in) grown in Cumalikizik village of Bursa province were used (Fig. 2).

Fig. 1. Coordinates of Cumalikizik village of Bursa province in Türkiye where *Ribes* genotypes were collected.

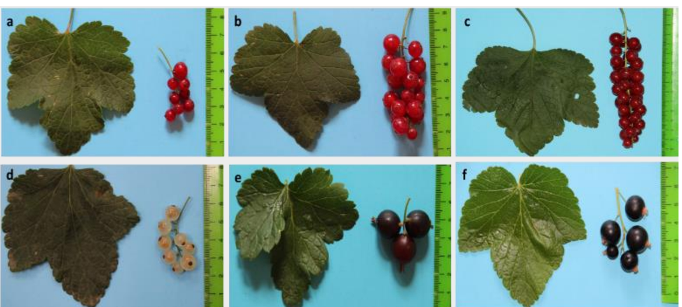

Fig. 2. Images of *R. rubrum* (a=RR1, b=RR2, c=RR3, d=RR4) and *R. nigrum* (e=RN1, f=RN2).

2.2. Physico-chemical analysis and physical properties

Two hundred of fruits were homogenized using a blender.

Total soluble solids and pH were determined using the methods outlined in the Association of Official Analytical Chemists, Official Method 932.12 (AOAC, 2000) and TS 1728 ISO 1842, respectively (TSE, 2001).

Berry diameter, berry height, and bunch height were measured with a digital caliper, and berries were weighed with a regular lab scale.

2.3. Color analysis

The color values of fruit samples were measured using a Chroma Meter (Konica Minolta CR-400, Osaka, Japan), and the CIE system was used to determine color. Color values were determined via calculation of the average of the reflectance values at three different points on each fruit.

2.4. Mineral content and bioaccessibility

Solutions in this analysis were prepared with ultrapure water with 18 MΩ.cm resistance using a water purification device (TKA Ultra Pacific and Genpura). HNO_3 (67%) was used (Merck, Darmstadt, Germany) and carrier gas was chosen as Argon (99.9995% purity, Linde, Türkiye). Standard stock solutions for each element were prepared in 1000 mg/L concentration to comply with Merck calibration standards (Darmstadt, Germany) using HNO_3 (0.3%).

The experimental method was validated by using Certified Cabbage: IAEA - 359 Austria, Certified Strawberry LGC7162 UK, Certified Tea NCSZC 73014 - (GSB-7) China. Lithium (Li), Cerium (Ce), Thallium (Tl), Yttrium (Y), and Cobalt (Co) were utilized as external standard solutions at 10 μg L^{-1}. The digestion process of the samples was carried out with a microwave digestion system (Milestone Brand MLS 1200 Mega, Italy). For disinfection of the containers, 10% HNO_3 (67% v/v), ultra-pure water, and oven-dried at 40°C were applied, respectively.

Table 1
Operating conditions of the ICP-OES.

Parameter	Value
Instrument	Optima 2100 DV
Detector	CCD detector
Nebulizer	Concentric
RF generator	40 MHz
RF power	1300 W
Plasma gas flow rate	15.0 L/min
Auxiliary gas flow rate	0.8 mL/min
Nebulizer gas flow rate	0.5 L/min
Pump speed	15 rpm
Auxiliary flow rate	1.0 L/min
Integration mode	Field
Wavelengths	Ca 317.933. nm; Cu 324.754 nm; Fe 238.204 nm; Mg 285.213 nm; Mn 257.610 nm; K 766.490 nm; Zn 213.856 nm (standard BS EN 16943:2017 Foodstuffs)

Samples (0.5 g) were homogenized and placed in each container and a mixture consisting of H_2O_2 (35%, 1 mL) and HNO_3 (65%, 6 mL) was added. A microwave burner (Milestone Brand MLS 1200 Mega) was used for digesting the samples in a five-step program (250 W/2 min, 0 W/2 min, 250 W/6 min, 400 W/5 min, and 600 W/5 min). After digestion, ultra-pure water (Millipore Milli-Q, 18.2 MΩ.cm resistant) was added to each sample up to 25 ml and then filtered (Hydropinilic PVDF Millipore Millex-HV, 0.45 μm). ICP-OES (TS EN 13805) was used for analyzing the filtered samples. Table 1 presents the operating conditions of the ICP-OES device and standard solutions were diluted from the stock solution (1000 mg L^{-1}) for the analyses. Besides, recovery studies, detection (LOD), and quantification (LOQ) limits were determined. At least six parallel samples were prepared and analyzed in three replicates. Recovery values were found out from the results indicated in Table 2. ICP-OES (Perkin Elmer 2100 USA) was used to determine the amount of K, Ca, Mg, Fe, Cu, Zn, and Mn for each sample. The results were expressed as mg per kg fresh weight.

Table 2
Performance characteristics of the method.

Element	LOD (mg/kg)	LOQ (mg/kg)	Recovery %
K	2.2	7.4	105
Ca	2.7	9.1	91
Mg	2.1	6.9	75
Fe	0.3	1.0	77
Cu	0.2	0.7	100
Zn	0.3	0.8	84
Mn	0.1	0.4	90

An artificial stomach and intestinal system were created sequentially for bioaccessibility studies with *in vitro* gastrointestinal extraction (Vitali et al., 2009). The bioaccessibility ratio was calculated as below;

Bioaccessibility %= [The value of the mineral content of the fruit after *in vitro* gastrointestinal extraction (mg kg^{-1}) / the value of the total mineral content of the fruit (mg kg^{-1})] x 100

2.5. Statistical analyses

At least three trees of each genotype were used, and six parallel samples were used for the parameters. Therefore, both biological and technical replicates were used for statistical analyses. Three ICP-OES measurements were performed for extracted samples. SPSS 23.0 software package was used for the statistical data evaluation. Statistically different groups between the average values obtained by the LSD test at a p-value of less than 0.05 ($p<0.05$).

3. Results and discussion

Significant variations ($p\leq0.05$) in physical and chemical properties were obtained between the genotypes of *R. rubrum* and *R. nigrum* (Tables 3, 4 and 5). These differences may be influenced by various factors including genetic, maturity, phenotypic differences, growing conditions and agricultural practices. In terms of average fruit sizes, RN1 had the biggest (16.13 mm in diameter,18.05 mm in height, and 2.5 g in weight) and RR1 had the smallest (7.59 mm in diameter, 7.56 mm in height and 0.32 g) berries. Kowalski and Gonzalez de Mejia (2021) evaluated ten blackcurrant variates and berry weights ranged between 0.47 to 1.22 g and diameters from 7.42 to 14.42 mm. This means *R. nigrum* genotypes have bigger berries compared to *R. rubrum* in general. Bunch lengths of RR3 and RR2 (150 and 113 mm respectively) were significantly greater than all others and RN1 (49 mm) had the shortest bunch length. The average number of berries per bunch was the highest in RR3 (32) and the lowest in RN1 (4). Seed numbers were also signifi-

Table 3
Fruit characteristics of *R. rubrum* (RR1-RR4) and *R. nigrum* (RN1 and RN2) genotypes.

Ribes genotypes	Berry diameter (mm)	Berry height (mm)	Berry weight (g)	Bunch length (mm)	Berry number in a bunch (number)	Seed number (number/fruit)	Total soluble solid (°Brix)	pH	Color		
									L*	a*	b*
RR1	7.59±0.52^{e}	7.56±0.56^{e}	0.32±0.06^{e}	52.14±4.88^{d}	7.43±1.82^{c}	3.00±1.00^{c}	9.13±0.06^{e}	2.83±0.04^{a}	26.13±1.69^{b}	19.64±2.38^{b}	9.60±1.90^{c}
RR2	10.32±0.52^{d}	9.66±0.38^{d}	0.67±0.12^{d}	113.00±12.55^{b}	21.20±7.33^{b}	4.00±1.83^{c}	7.73±0.06^{f}	2.77±0.01^{ab}	24.06±1.79^{c}	24.45±3.09^{a}	12.35±3.28^{b}
RR3	12.63±0.56^{c}	11.43±0.54^{c}	1.28±0.04^{c}	150.00±35.78^{a}	32.17±13.12^{a}	10.75±2.75^{b}	12.07±0.15^{c}	2.70±0.04^{b}	21.71±1.32^{d}	19.16±3.24^{b}	8.30±1.93^{c}
RR4	9.93±0.85^{d}	9.13±0.55^{d}	0.60±0.08^{d}	72.92±16.02^{c}	8.40±1.96^{c}	4.67±1.03^{c}	10.77±0.06^{d}	2.69±0.01^{b}	42.28±1.60^{a}	5.10±1.02^{c}	16.06±1.71^{a}
RN1	16.13±0.48^{a}	18.05±1.24^{a}	2.50±0.46^{a}	49.00±4.8^{d}	4.00±0.71^{d}	9.11±5.69^{b}	12.57±0.40^{b}	2.80±0.09^{a}	23.86±0.98^{c}	2.73±1.27^{d}	0.77±0.46^{d}
RN2	13.64±0.47^{b}	12.94±0.68^{b}	1.56±0.21^{b}	51.00±6.52^{d}	4.60±0.55^{d}	26.90±8.18^{a}	13.40±0.00^{a}	2.86±0.00^{a}	24.50±1.25^{c}	0.15±0.11^{e}	0.05±0.19^{d}

Different letters in the same lines represent results with statistical difference, according to the Fisher's LSD test ($p \leq 0.05$).

cantly differed between the genotypes. Among six genotypes RN2 had a significantly greater seed number (27 approximately) while RR1 had the lowest (3). In general, greater values in berry diameter, height, weight, and seed number were recorded in *R. nigrum* genotypes. On the other hand, *R. rubrum* genotypes were superior in bunch length and berry number. Studies also declare that environmental factors can affect fruit sizes dramatically, although genotypes rankings tend to stay consistent (Brennan et al., 2008; Kaldmäe et al., 2013).

Significant differences observed in soluble solids (ºBrix, $p \leq 0.05$) between *R. rubrum* and *R. nigrum* genotypes. RN2 showed the highest values (13.40 ◦Brix) in terms of soluble solid contents while the lowest was in RR2 (7.73 ◦Brix). These results are consistent with results obtained in varieties of Netherlands, Scotland, England, Germany, etc. origin presented by Zdunić (2016). Soluble solid contents in *R. nigrum* were reported to range from 13.89 ◦Brix to 16.14 ◦Brix (Rubinskiene et al., 2005; Contessa et al., 2013) and between 7.40-10.70 ◦Brix in the *R. rubrum* varieties (Pantelidis et al., 2007). According to Clark et al., (2018), soluble solid content of up to 24 ºBrix was reported among blackcurrant selections but the mean was 14 ºBrix. Likewise, soluble solid content for some blackcurrant varieties was reported as 20.80°Brix in the study of de Souza et al. (2014).

Acidity is another factor that may affect the consumer's preferences and a significant difference was found between *R. rubrum* and *R. nigrum* genotypes in terms of pH values which ranged from 2.86 (RN2) to 2.69 (RR4) in the present study. In good agreement with our findings, Kowalski and Gonzalez de Mejia (2021) reported the pH values of ten blackcurrants between 2.80 to 2.96. On the other hand, the pH of a newly introduced blackcurrant variety *R. anatolica* Behcet was determined as 4.12 in the study of Yurt et al. (2021).

We observed that *R. rubrum* and *R. nigrum* species differed in color (Fig. 2, Table 3). Significant differences in L* and b* values were recorded in RR4 (42.28 and 16.06, respectively), and a* value (24.45) was recorded in RR2 among the detected currant species. These results are already expected because RR4 has the lightest color among all genotypes. *R. rubrum* genotypes had relatively higher a* values indicating that those berries reflected red color more than *R. nigrum* genotypes. Eksi Karagac et al., (2020) reported that a red currant, the 'Red Lake' cultivar showed higher values according to other varieties, with 32.88, 19.40, and 8.04 for L*, b*, and a*. In addition, *R. anatolica* Behcet cultivar L*, a*, and b* values were determined as 57.79, 10.19, and 4.03, respectively.

Three minerals, namely K, Ca, and Mg, were prominent in the blackcurrant genotypes in this study. The K was determined as the most prevalent element among the samples and the amounts of minerals decreased with the following order: Ca, Mg, Fe, Cu, Zn, and Mn (Table 4). Our finding is in line with Cosmulescu et al. (2015), who reported that K was the most abundant mineral in their analyses of black and red currants. In the present study, the order of nutritive element contents/100 g of fruits was presented as K > Ca > Mg > Fe > Al > Na > Mn > B > Cu. In another study about the mineral content of 7 black currants cultivars, among them cultivar named the "Ben Sarek" had the highest amount of K (330.90-327.10 mg/100g) and the mineral contents of those cultivars were ranked in the following order: K > P > Ca > Mg > Na > Fe > Cu > Zn > Se > Mn (Paunovic et al., 2017). Significant differences were observed ($p \leq 0.01$) in terms of the concentration of minerals between *Ribes* genotypes cultivated in the Cumalikizik village of Bursa. It is thought that these variations in the mineral contents of genotypes are related to the properties that are inherent to each genotype because all of them were grown in the same climate and soil conditions. K levels ranged from 2215,4 mg kg^{-1} (RN1) to 1875.50 mg kg^{-1} (RR3) in the samples. According to Eksi Karaagac et al. (2020) K concentration of the Red Lake variety was 10205.91 mg kg^{-1} dw. Compared to this study greater K concentrations obtained in our study that consists of per dry weight evaluation.

The highest value of Ca was determined in RR1 (603.10 mg kg^{-1}), followed by RN1 (403.40 mg kg^{-1}) and RN2 (383.90 mg kg^{-1}). Fe content was the highest in the RR4 genotype as 10.30 mg kg^{-1}, while the lowest Fe content was determined in RR3 as 5.1 mg kg^{-1}. In addition, Mn and Mg contents were the highest in the RN2 genotype with 1.90 mg kg^{-1} and 212.90 mg kg^{-1}, respectively. Cu content was found the highest in RN1 as 2.80 mg kg^{-1}. Moreover, the highest value in Zn content was detected in RR1 as 4.70 mg kg^{-1}, followed by *R. nigrum* genotypes (2.60 mg kg^{-1} for RN1 and 2.40 mg kg^{-1} for RN2). K and Mg contents in our study were lower than those reported by Nour et al. (2011). K, Mg, and Cu contents found in our study were also lower than those reported by Paunović et al. (2017), while Ca and Mn contents found in our study were higher. These differences might have arisen due to the differences in weather,

Table 4
Mineral contents of *R. rubrum* (RR1-RR4) and *R. nigrum* (RN1 and RN2) genotypes.

Ribes genotypes	K	Ca	Fe	Mn	Cu	Zn	Mg
RR1	2068.80±44.10^{ab}	603.10±29.50^{a}	6.80±0.20^{d}	1.30±0.10^{bc}	2.20±0.10^{bc}	4.70±1.50^{a}	198.20±3.30^{b}
RR2	1997.20±111.20^{bc}	304.30±33.10^{c}	8.00±0.10^{c}	1.00±0.00^{d}	1.20±0.00^{e}	1.30±0.10^{c}	136.10±5.10^{d}
RR3	1875.50±4.50^{c}	296.80±19.20^{c}	5.10±0.20^{e}	1.20±0.00^{cd}	2.30±0.00^{b}	1.20±0.20^{c}	117.90±0.30^{e}
RR4	2208.10±42.00^{a}	200.40±16.40^{d}	10.30±0.40^{a}	1.20±0.20^{cd}	1.30±0.00^{d}	1.20±0.10^{c}	155.90±3.30^{c}
Mean	**2037.40±137.30**	**351.20±162.70**	**7.50±2.00**	**1.20±0.20**	**1.80±0.50**	**2.10±1.70**	**152.10±32.00**
RN1	2215.40±82.60^{a}	403.40±26.10^{b}	9.40±0.10^{b}	1.50±0.00^{b}	2.80±0.10^{a}	2.60±0.50^{b}	158.30±3.20^{c}
RN2	2188.20±62.10^{a}	383.90±0.00^{b}	7.80±0.40^{c}	1.90±0.10^{a}	2.10±0.10^{c}	2.40±0.10^{b}	212.90±1.40^{a}
Mean	**2201.80±61.70**	**393.70±18.80**	**8.60±1.00**	**1.70±0.20**	**2.40±0.40**	**2.50±0.30**	**185.60±31.60**

Data were presented as means ± standard deviations; values were expressed as mg kg^{-1} fresh weight for minerals. Different letters in the same lines represent results with statistical difference, according to the Fisher's LSD test ($p \leq 0.05$).

soil, and cultivation conditions which the plants were grown. Nevertheless, the mineral contents reported in the present study do not yield fold differences when compared with relevant reports in the literature. The average mineral analysis of in genotypes of *R. nigrum* and *R. rubrum* revealed no differences between genotypes for both species except for Mn content. Black currants were superior to red currants regarding Mn contents. A previous study reported similar contents for K, Ca, and Mn while Mg content was higher in *R. nigrum* and Fe content was higher in *R. rubrum* (Eksi Karaagac et al., 2020).

Ribes species were found to contain higher K, Mg, Ca, and Cu content compared to other berries like strawberry, raspberry blackberry, blueberry, and (Karlsons et al., 2018; Pereira et al., 2018). In addition, K, Mg, Ca, Fe, and Zn contents of Ribes species were also reported to be higher than those in other fruits like apple and pear (Turkkomp, 2024; USDA, 2024). In accordance, our analyses suggest that *Ribes* species is a good source of minerals.

Table 5
Bioaccessibility (%) of the mineral contents of *R. rubrum* (RR1-RR4) and *R. nigrum* (RN1 and RN2) genotypes.

Ribes genotypes	K	Ca	Fe	Mn	Cu	Zn	Mg
RR1	66%	47%	28%	45%	31%	43%	42%
RR2	71%	98%	37%	56%	54%	16%	64%
RR3	95%	95%	40%	63%	39%	18%	72%
RR4	89%	97%	21%	50%	42%	17%	54%
Mean	80%	84%	32%	54%	42%	24%	58%
RN1	91%	72%	28%	48%	25%	84%	66%
RN2	95%	97%	24%	38%	29%	88%	82%
Mean	93%	85%	26%	43%	27%	86%	74%

However, the total amount of the mineral content is not always correlated with real nutritional values since the whole digested amount of nutrients is not completely absorbed (Usal and Sahan, 2020). Thus, a compound with high content in a plant product might have low bioaccessibility value. Therefore, research on the chemical characterization of plants must be accompanied by bioaccessibility studies to estimate their health benefits. In this study, bioaccessibility values (%) of the mineral contents of *R. rubrum* (RR1-RR4) and *R. nigrum* (RN1 and RN2) genotypes were presented in Table 5. The mineral bioaccessibility ranged from 17 to 98% in the present study. The minerals were aligned as per bioaccessibility from high to low as K (71-95 %), Ca (47-98%), Mg (42-82%), Zn (17-88%), Mn (38-63%), Cu (27-54%) and Fe (21-40%) for all genotypes.

RN1 genotype had the highest K concentration but its bioaccessibility was lower (91%) than those of RR3 and RN2 (95% for all). Similarly, Ca concentration was the highest in RR1 with 603.10 mg kg^{-1} but was the lowest in bioaccessibility with 47% among all genotypes. Mg bioaccessibility was the highest in RN2 with 82% (212.90 mg kg^{-1}) but lowest in RR1 with 42% even the Mg
content was measured as 198.2 mg kg^{-1}. When the two *Ribes* species were compared, bioaccessibilities of Cu, Fe, and Mn were higher in *R. rubrum* and bioaccessibilities of K, Ca, Zn, and Mg were higher in *R. nigrum*. A striking difference concerning average bioaccessibility of genotypes was noted for Zn which was analyzed as 24% in *R. rubrum* and 86% in *R. nigrum*.

The highest bioaccessibility on average was detected for K in both *Ribes* species. A previous study reported that the average value of K bioaccessibility in fruits and vegetables was approximately 67%, specifically 64% in fruits (avocado, banana, and kiwifruit), and 72% in vegetables (Ceccanti et al., 2022). Thus, results of our study show that both the content and the bioaccessibility of K might be greater in *Ribes* species than those in most fruit and vegetables. Therefore, *Ribes* species could be considered as a good K source. It has been proposed that the indigestible cell walls found in plants could form a physical barrier that reduces the intracellular nutrients' bioaccessibility (Naismith and Braschi, 2008). Therefore, their high content of mineral substances but their low bioaccessibility in fruit and vegetables may cause an overestimation of the contribution of fruits or vegetables to the diet. When it comes to mineral intake, the comparatively low bioaccessibility of minerals reduces the perceived nutritional value of food. The relatively poor bioaccessibility of minerals reduces the nutritive value of foods with regard to mineral intake.

According to Larsson et al. (2008), a high intake of minerals like K, Mg, and Ca has a direct connection to reduced risk of stroke, hypertension, and osteoporosis. FDA updated the intake of daily values of minerals and there are increases and decreases for some minerals. For example, Ca daily value (DV) increased from 1000 to 1300 mg, K DV increased from 3500 to 4700 mg, Mg DV increased from 400 to 420 mg, and Mn DV from 2 to 2.3 mg. Cu and Zn DV decreased from 15 to 11 mg and 2 to 0.9 mg respectively. Fe DV remains the same as 18 mg before (FDA, 2021). Therefore, knowledge of the amount and bioaccessibility of minerals in fruits can be decisive in guiding the consumer's preferences.

According to the results of mineral content analyses and bioaccessibility results, the investigated *Ribes* species were found as a potential source of K, Ca, and Mg minerals. Although bioaccessibilities of bioactive compounds of *Ribes* were reported previously (Trych et al., 2022), to our knowledge this is the first study to provide data on bioaccessibilities of the mineral contents of *Ribes*.

4. Conclusion

Since breeders continuously need to monitor crops for phenotypic variations from a large genetic pool it is necessary to analyze the physical, chemical, and nutritional characteristics of the genotypes presented in these studies.

In this study, six *Ribes* genotypes (RR1-RR4 and RN1-RN2) were examined in terms of their biophysicochemical properties, mineral content, and bioaccessibility, providing required data for breeding studies. According to the results, compared to other minerals K, Ca, and Mg are abundant in *Ribes* genotypes.

On the other hand, *R. nigrum* genotypes consist of higher mineral levels and better quantified physical properties than *R. rubrum* genotypes. Consequently, this study does not only reveal promising varieties for cultivation but also can present useful guidance for future studies.

Conflict of interest: The authors declare that they have no conflict of interests.

Informed consent: The authors declare that this manuscript did not involve human or animal participants and informed consent was not collected.

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Research article

Analysis of antioxidant capacity, total phenolic and total flavonoid contents in boric acid applied *Camellia sinensis* L.

Isil Sezekler[*1]

[1] *Marmara University, Faculty of Science, Department of Biology, 34722, Istanbul, Türkiye*

Abstract

Consumption of *Camellia sinensis* L. (tea), a popular beverage, is very common today. In addition to its consumption as a beverage, it is suggested that adding tea to other foods can increase their antioxidant activities. It is known that boric acid used as an insecticide, herbicide, and fungicide, has antioxidant and anti-inflammatory effects. This study aimed to determine the antioxidant capacity (AC), total phenolic content (TPC), and total flavonoid content (TFC) of extracts prepared in different solvents of tea grown in soil treated with boric acid. The area in Rize/Türkiye was divided into 4 groups. No application was made to the control group (B0 group). Boric acid prepared in sodium tetraborate buffer was applied to the other three areas as a single dose at concentrations of 100 (group B1), 300 (group B3), and 500 (group B5) mg m^{-2}. The obtained tea leaves were ground and infused in water, 20% ethanol, and 50% ethanol by the classical infusion method. The extracts obtained after infusion were analyzed for TPC, TFC, and AC. It was determined that ethanol (20% and 50%) was better solvent than water in terms of TPC, TFC, and AC. Although the TPC of extracts prepared in water of tea leaves grown in soils where different doses of boric acid were applied did not change, differences were observed in their flavonoid contents and antioxidant capacities. These results indicated that boric acid should be at a certain dose to improve the quality of the tea plant. In addition, different solvents can be used to reveal more of the tea content.

Keywords: *Antioxidant capacity; boric acid; C. sinensis; flavonoid content; green tea; phenolic content*

1. Introduction

Camellia sinensis L. (tea) is a plant greatly cultivated in tropical and subtropical regions and is the most widely consumed beverage after water due to its cooling, revitalizing, and mild stimulant effects containing highly bioactive compounds beneficial to health. These bioactive compounds in the tea plant include alkaloids, flavonoids, triterpenoids, caffeine, steroids, saponins, oils, carotene, essential oils, and vitamins (Ramlah, 2017). In addition, tea has bioactive properties such as antioxidative, anti-bacterial, anti-viral, and anti-inflammatory. Tea is also greatly used as a component of folk medicines (Hayat et al., 2015; Zhao et al., 2022).

Nowadays, the use of tea has started to change and a lot of research and development has been done on food products enriched with tea powder, including sponge cake (Ahmed et al., 2023), biscuits (Phongnarisorn et al., 2018), bread (Ning et al., 2017), dry noodles (Yu et al., 2020) and ice cream products (Baruah et al., 2012). The addition of green tea powder to food products can enhance antioxidant activity and significantly reduce peroxide production during product storage (Ning et al., 2017). Phenolic compounds, flavonoids, and antioxidants are the dominant constituents of tea leaves, accounting for 30% of their dry weight. Antioxidants protect living organisms from uncontrolled production of reactive oxygen species and accompanying protein, lipid, and DNA damage (Srinivasan et al., 2007; Juan et al., 2021). Antioxidant compounds such as polyphenols, phenolic acids, and flavonoids scavenge free radicals such as peroxide, hydroperoxide, and/or lipid peroxide and thus inhibit oxidative mechanisms leading to degenerative

[*] Corresponding author.
E-mail address: isilismailoglu@gmail.com (I. Sezekler).
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diseases (Wu et al., 2011). Due to its diverse composition and benefits, tea can be used in different sectors, most notably the food processing sector. Several substances can be added to improve the color, taste, shape, and texture of tea and to extend its shelf life, in addition, adding green tea powder to baked goods is also beneficial for health (Goh et al., 2015; Mashkour et al., 2022). According to the study by Owuor et al. (2008), compounds that have beneficial health effects in tea are affected by many factors such as variety, harvest season, plant age, climate, environmental conditions, and processing conditions. Catechins and epigallocatechin gallate are the most abundant phenolic and flavonoid compounds in tea, and thanks to these compounds, tea shows antioxidant activity (Liang et al., 2024; Ntamo et al., 2024).

Boron is one of the essential microelements in plant growth. In addition, it has many different functions such as tissue differentiation, vegetative growth, membrane integrity, regulation of enzymatic reactions, sugar transport, and nucleic acid synthesis (Camacho-Cristobal et al., 2008; Koshiba et al., 2009; Ahmad et al., 2012; Pereira et al., 2021). Boron, one of the important elements involved in the concentration and metabolism of phenolic compounds in plants, should be present in soil and irrigation water under natural conditions. Deficiency or toxicity of boron in the form of boric acid in the soil in agricultural areas affects product yield and quality. Moreover, boron deficiency leads to oxidative stress and cell death. There is a decrease in antioxidant activity in the roots and leaves of plants with boron deficiency. Research has shown that high boron concentration increases antioxidant enzyme activity and thus protects against oxidative stress (Balci and Taban, 2023).

In this study, it was aimed to explore the concentrations of the TPC, TFC, and AC of the extracts obtained by infusing tea leaves grown in soils treated with different concentrations of boric acid in different solvents. Thus, this study aims to increase the antioxidant properties of tea leaves by improving crop quality during tea cultivation and to maximize the beneficial effects of natural antioxidant intake with consumption.

2. Materials and methods

2.1. Collection of experimental material

Fresh leaves of *C. sinensis* were collected from the Caykur trial garden located in the Rize/Cayeli district. The plot was divided into four groups. Each group consisted of five different areas ($10m^2$). While no application was made to the fields in the control (Boron 0) group, boric acid prepared in sodium tetraborate buffer at 3 different concentrations (Boric acid 1 (B1)-100 mg m^{-2}, Boric acid 3 (B3)-300 mg m^{-2}, and Boric acid 5 (B5)-500 mg m^{-2}) was applied to the soil of the other three groups. Harvested tea plant leaves were dried at room temperature (RT) for a week, ground with a grinding machine, and stored at room temperature (RT).

2.2. The preparation of C. sinensis leaf extract

For the extracts to be used in the study, the classical infusion method was used for the ground tea samples. In the classical infusion method, water, 20% ethanol, and 50% ethanol were used as solvents for each experimental group. To infuse 5 g of tea leaves, 250 mL of liquid was used at 30°C. The extracts obtained were cooled to RT and centrifuged at 4000 g for 15 min and then, the supernatants were used for TPC, TFC, and AC analysis.

2.3. Total phenolic content (TPC) analysis

Quantification of TPC was carried out by spectrophotometric method. For the detection of the concentration of TPC, 1500 µL Folin-Ciocalteu reagent (2 N, diluted 10 times) was added to the tubes containing 300 µL blank, standard compound gallic acid (GA) and tea leaf extract, and the solutions to be mixed thoroughly were kept in the dark at RT for 3 min. Then, 1.2 mL of 7.5% (w/v) Na_2CO_3 was added to the extract, standard, and blank tubes mixed thoroughly, and incubated for 90 min at RT and in the dark. After incubation, absorbances were read at 765 nm. The concentration of TPC in the extracts was calculated using the standard calibration chart created with OD765 values of different GA concentrations (Singleton and Rossi, 1965). The TPC concentration was expressed as µg GA equivalent/mL.

2.4. Total flavonoid content (TFC) analysis

The TFC of tea leaf extract was analyzed by the $AlCl_3$ colorimetric method (Zhishen et al., 1999). In the method, 4% NaOH, 10% $AlCl_3$, and 5% $NaNO_2$ solutions were used. Quercetin was used to prepare the calibration curve. For flavonoid determination, 4 mL distilled water, 1 mL standard quercetin solutions, and tea leaf extract were added to the tubes, and 0.3 mL 5% $NaNO_3$ was added and incubated for 5 min at RT. After incubation, 0.3 mL of 10% $AlCl_3$ was added to the tubes and kept in the dark and at RT for 5 min. Then, 2 mL of 4% NaOH and 2.4 mL of dH_2O were added to the tubes and absorbance measurements were performed at 415 nm. The concentration of total flavonoids in the extracts was calculated by using the standard calibration graph of quercetin.

2.5. Determination of antioxidant capacity (AC)

The total AC of tea extracts was detected according to Prieto's method (Prieto et al., 1999). 0.4 mL of tea leaf extracts were taken and 4 mL of phosphomolybdenum solution (obtained by mixing 0.6 M H_2SO_4, 28 mM $Na_2HPO_4.12H_2O$ and 4 mM ammonium molybdate, 25 mL each) was added, the analysis tubes were well closed with leak-proof caps, vortexed and incubated in a water bath at 95°C for 90 min. Subsequently, the samples were rapidly cooled, and the absorbance values of the green color formed after the reaction were determined at 695 nm. The ascorbic acid standard solution was prepared for the calculation. AC was presented as ascorbic acid equivalent (mg AAE/g) dry extract.

2.6. Statistical analysis

GraphPad Prism Version 9.0 statistical software was used for data analysis. The results are presented as mean ± SD. Data distributions were based on the Shapiro-Wilk test. ANOVA and Tukey's post hoc tests were used for analyses. P value less than 0.05 was accepted as significant.

3. Results

3.1. Total phenolic content (TPC)

There was no difference in the TPC of the extracts obtained

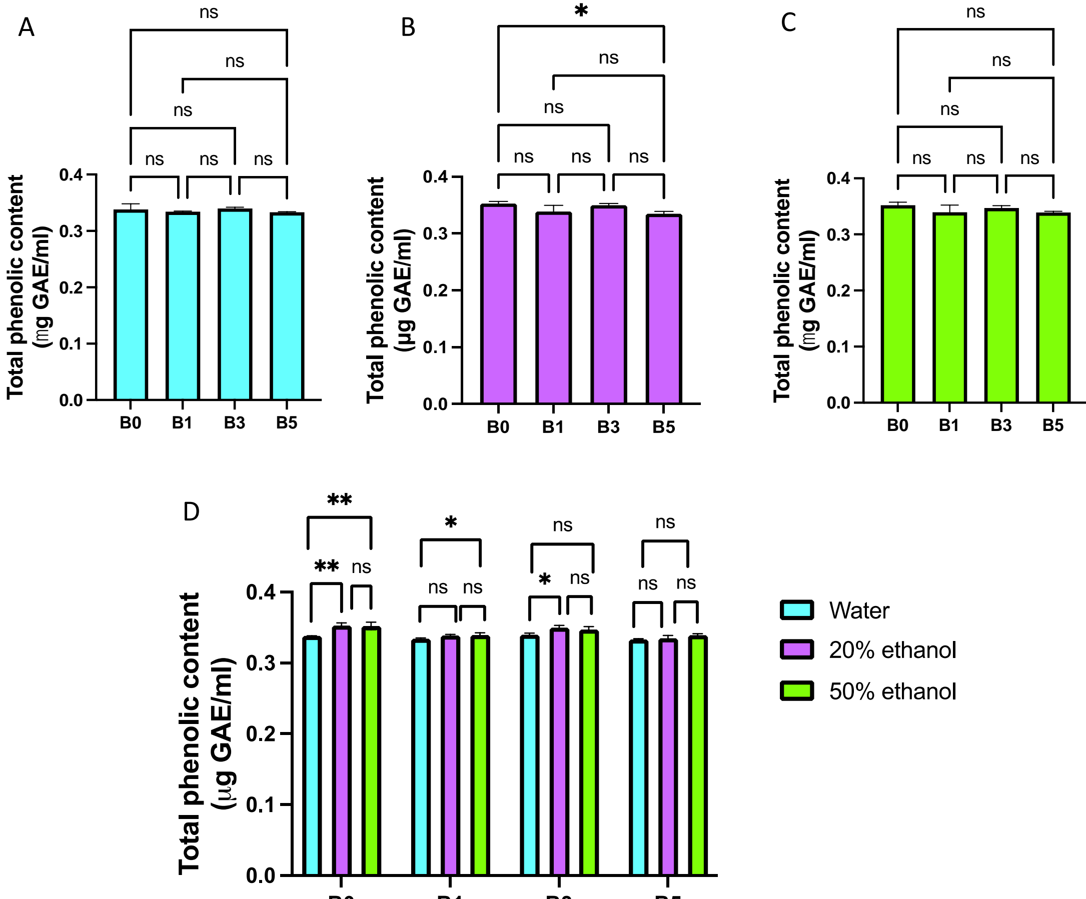

Fig. 1. Effects of 0 mg m^{-2} (B0), 100 mg m^{-2} (B1), 300 mg m^{-2} (B3), 500 mg m^{-2} (B5) boric acid treatments on TPC. Tea extract prepared with water (A), tea extract prepared with 20% ethanol (B), tea extract prepared with 50% ethanol (C), comparison of total phenolic compounds of different solvents (D). *$p < 0.05$, **$p < 0.01$, ns; non-significant.

from water infusion of tea leaves grown in soils with different doses of boric acid (Fig.1A). The concentration of TPC in the extract prepared with 20% ethanol of tea leaves collected from the B1 and B3 groups did not show any change compared to the B0 group. The concentration of TPC in the extract prepared with 20% ethanol of tea leaves in the B5 group was significantly decreased compared to the B0 ($p < 0.05$, Fig. 1B). Similar to the tea extracts prepared with water, no difference was observed in the TPC concentration of the tea extracts prepared in 50% ethanol among the B0, B1, B3, and B5 groups (Fig. 1C).

The TPC concentration of the extracts obtained by infusing tea leaves grown in soil treated with boric acid at different concentrations in different solvents is shown in Fig. 1D. It was determined that the TPC concentration of tea extracts obtained by brewing in 20% and 50% ethanol of tea leaves grown in non-boric acid treated soils was significantly increased compared to tea extracts prepared with water ($p < 0.01$ for both). The TPC concentration of the extracts prepared with 50% ethanol of the teas grown in group B1 was higher than the extracts prepared with water ($p < 0.05$). The TPC concentration of the extracts prepared with 20% ethanol of the teas grown in the B3 group was significantly higher than the extracts prepared in water ($p < 0.05$). However, there was no difference in the amount of TPC between the extracts prepared with different solvents of the B3 group.

3.2. Total flavonoid content (TFC)

The TFC of tea extracts prepared with water was significantly lower in the B1 and B5 groups compared with the B0 group ($p < 0.001$ and $p < 0.0001$, respectively, Fig. 2A). When the B1 and B5 groups were compared with the B3 group, the TFC of tea extracts prepared with water was lower in the B1 and B5 groups ($p < 0.0001$ for both). Similar to water, the TFC of tea extracts prepared with 20% ethanol was lower in the B1 and B5 groups compared to the B0 group ($p < 0.01$ for both). The TFC in the B3 group was significantly higher compared to the B1 and B5 groups ($p < 0.05$ and $p < 0.01$, respectively, Fig. 2B). The TFC of tea extracts prepared with 50% ethanol was significantly decreased in the B1, B3, and B5 groups compared to the B0 group ($p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively, Fig. 2C).

The TFC of the extracts obtained from tea leaves grown in soil treated with boric acid at different concentrations and infused in different solvents are presented in Fig. 2D. The TFC of the tea extracts obtained by brewing the tea leaves collected from the B0 group in 20% and 50% ethanol were significantly increased compared to the tea extracts prepared with water ($p < 0.01$ for both). However, there was no difference between 20% and 50% ethanol extracts. The TFC in both 20% and 50% ethanol extracts of teas grown in group B1 were higher than those prepared with water ($p < 0.001$ and $p < 0.0001$, respectively). In the B1 group, the TFC of tea extracts prepared in 50% ethanol were higher than those prepared in 20% ethanol ($p < 0.001$). The TFC of tea extracts prepared in 20% and 50% ethanol was significantly higher than those prepared in water ($p < 0.05$ for both). Similarly, the TFC of extracts prepared in water of teas grown in group B5 was lower than those prepared in 20%

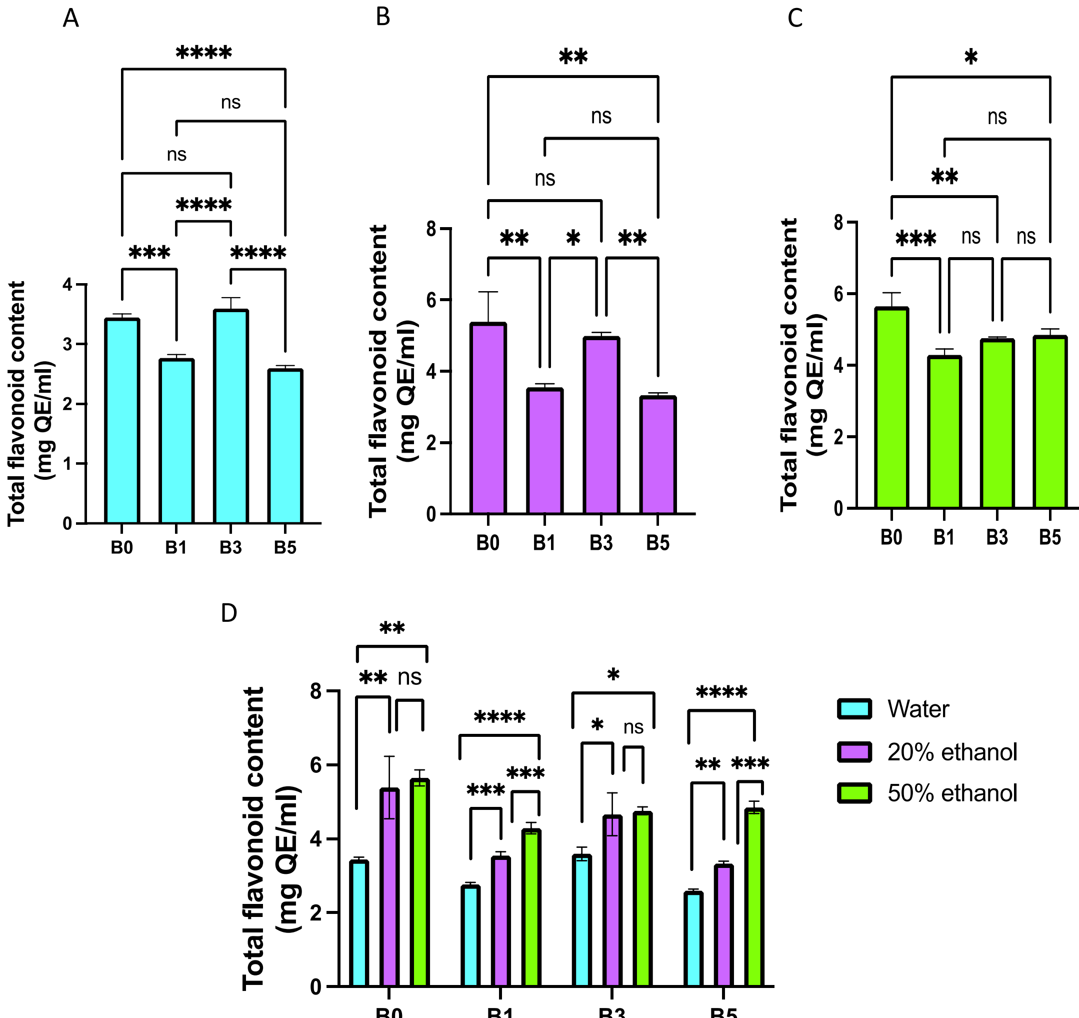

Fig. 2. Effects of 0 mg m^{-2} (B0), 100 mg m^{-2} (B1), 300 mg m^{-2} (B3), 500 mg m^{-2} (B5) boric acid treatments on the TFC in tea extract prepared with water (A), 20% ethanol (B), and 50% ethanol (C). Comparison of TFC in different solvents (D). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns; non-significant.

and 50% ethanol ($p < 0.01$ and $p < 0.0001$, respectively). In the B3 group, the TFC of tea extracts prepared with 50% ethanol was higher than those prepared with 20% ethanol ($p < 0.001$).

3.3. The antioxidant capacity (AC)

When the AC in the tea extracts prepared with water was compared, it was observed that the AC in the B1 and B5 groups was significantly lower compared to the B0 group ($p < 0.001$, $p < 0.0001$, respectively, Fig. 3A). However, the AC elevated in the B3 group compared to the B0 group ($p < 0.01$, Fig. 3A). Moreover, the AC in the B3 group was higher compared to the B1 and B5 groups ($p < 0.0001$ for both, Fig. 3A). The AC in the tea extracts prepared in 20% ethanol was similar to the brewed tea extracts, and the AC was lower in the B1 and B5 groups compared with the B0 group ($p < 0.0001$ for both, Fig. 3B). The AC in the tea extracts prepared with 50% ethanol in the B0 group was higher than in the B1, B3, and B5 groups ($p < 0.0001$, $p < 0.05$, and $p < 0.001$, respectively, Fig. 3B). The AC in the B1 and B5 groups was significantly reduced compared to the B3 group ($p < 0.01$ for both, Fig. 3C).

As the AC in the extracts prepared with different solvents of tea leaves grown in boron-free soils was compared, it was found that the AC of the extracts prepared in 20% and 50% ethanol were higher than those prepared in water ($p < 0.001$ for both, Fig. 3D). In the B1 group, the AC in the tea extracts brewed in 20% and 50% ethanol was significantly increased compared to the extract prepared in water ($p < 0.05$ and $p < 0.01$, respectively, Fig. 3D). Similarly, in group B3, the AC in tea extracts prepared in 20% and 50% ethanol was higher than the tea extract prepared in water ($p < 0.01$ for both, Fig. 3D). In group B5, the AC in the tea extract brewed in 20% ethanol was similar to that of extract brewed in water. However, the AC in the extract dissolved in 50% ethanol was higher than the tea extracts brewed in water and 20% ethanol ($p < 0.001$ for both, Fig. 3D).

4. Discussion

In this study, TPC, flavonoid content, and AC in the extracts prepared in water, 20% and 50% ethanol solvents from tea leaves grown in soil treated with different concentrations of boric acid were evaluated.

Tea has economic and social importance for many countries around the world. Consumption of tea, which is considered the second most consumed beverage in the world after water, is known to have beneficial effects on various human diseases such as cardiovascular and liver diseases (Keller and Wallace, 2021; Li et al., 2022). Tea is composed of polyphenols, minerals, caffeine, and trace amounts of amino acids, vitamins,

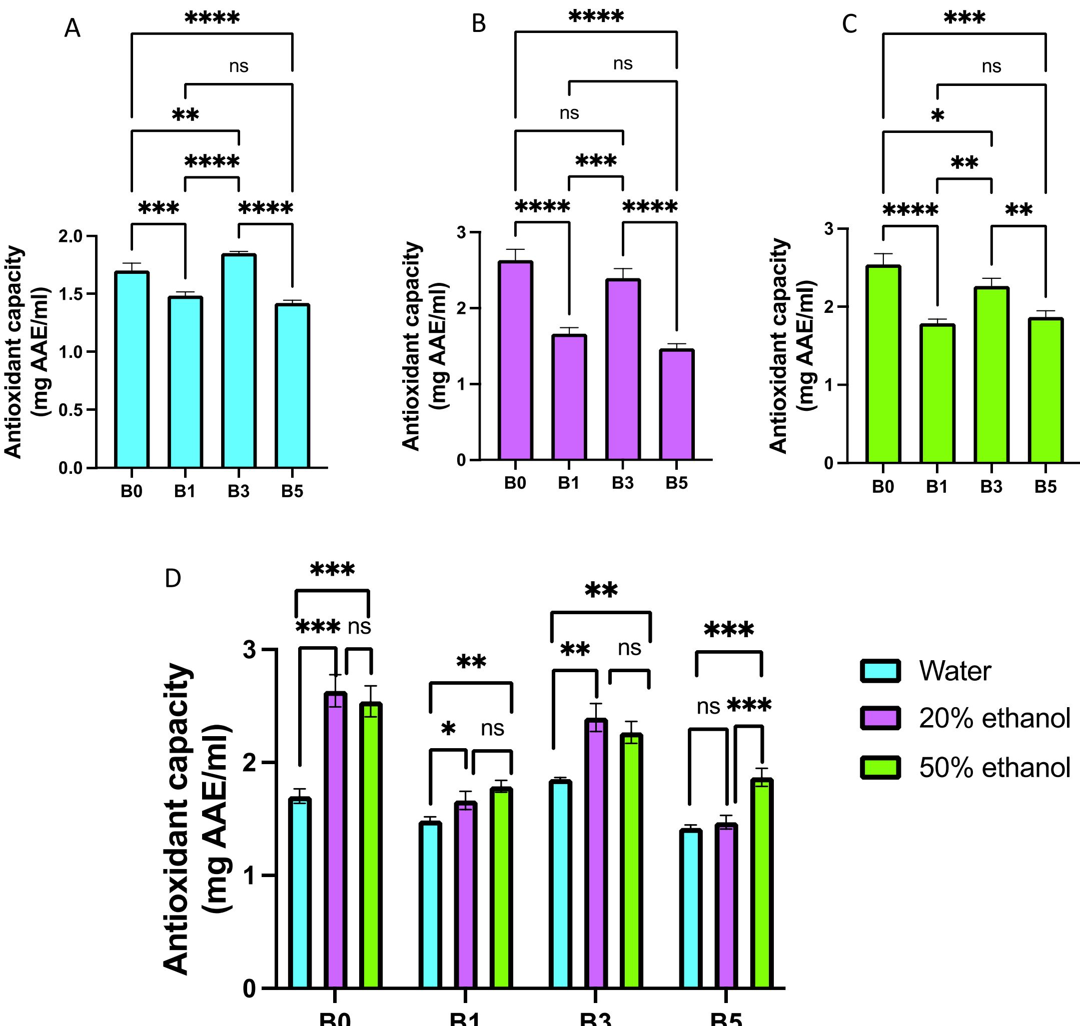

Fig. 3. Effects of 0 mg m^{-2} (B0), 100 mg m^{-2} (B1), 300 mg m^{-2} (B3), 500 mg m^{-2} (B5) boric acid treatments on the AC in tea extract prepared with water (A), 20% ethanol (B), and 50% ethanol (C). Comparison of AC in different solvents (D). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns; non-significant.

and carbohydrates, and the composition of tea varies depending on the fermentation process used for its production. Green tea has been noticed to be richer in antioxidants compared with other types of tea (Prasanth et al., 2019). The health benefits of green tea are associated with its high catechin level. In addition, the application of micronutrients such as boron, manganese, and zinc are important in increasing the yield of tea (Karak et al., 2017; Kumar, 2017).

It has been suggested that boron deficiency affects the growth of tea as well as its quality (Baruah et al., 2011). According to Gohain et al. (2000), foliar boric acid application had little effect on tea quality. Similarly, Hajiboland et al. (2013) reported that tea plant has a high tolerance to boron deficiency. In this study, it was determined that there was no difference in the concentration of TPC in the extracts prepared with water, 20%, and 50% ethanol from tea leaves grown in boric acid-treated soils. There was a decrease in the concentration of TPC in the extracts prepared with 20% ethanol from tea leaves grown in the B5 group. While the TFC level of the extracts prepared with water and 20% ethanol from tea leaves grown in the B0 and B3 groups were similar, a decrease was observed in the B1 and B5 groups compared to B0. The TFC level in the tea extract prepared with 50% ethanol was the highest in the B0 group. When the AC in the tea extracts was compared, the AC in the extract brewed in water was the highest in the B3 group. Also, the AC of the extracts prepared in 20% and 50% ethanol was the highest in the B0 group. While the application of boric acid to the soil does not affect the TPC concentration in tea leaves grown in this soil, its effect on the TFC concentration and AC varies depending on the dose of boric acid.

In studies on plant extracts, it was determined that TPC, TFC, and antioxidant activity values differed depending on solvents. It was emphasized that different solvents should be used for each plant (Coklar and Akbulut, 2016; Bursal et al., 2021; Yolci et al., 2022). Cabrera et al. (2021) examined green tea extracts prepared with synthetic fresh water, mineral salt solution, sodium bicarbonate solution, and deionized water at five different hardness levels to evaluate the effect of water hardness on caffeine and catechin content. As a result, they found that total catechin yield decreased as water hardness increased. Catechins, which have strong antioxidant properties, are polyphenol compounds that are important components of tea leaves as in many plants (Bae et al., 2020).

In the study by Baskaya Sezer (2023) on the phenolic contents of blackberry (*Rubus plicatus* L.) extracts prepared with different solvents (water, ethanol, methanol, and the forms of these solvents with acetic acid (1%) or hydrochloric acid (1%) added), it was reported that the highest phenolic content was found in samples extracted with ethanol and methanol with hydrochloric acid added. In another study, the TPC of yellow

and blue poppy (*Papaver somniferum* L.) seed extracts prepared with different solvents such as ethanol, methanol, purified water, and acetone were analyzed. For yellow and blue poppy, the highest concentration of phenolic content was reported in the extract prepared with ethanol. The lowest concentration of phenolic content was found in the extract obtained with acetone for yellow poppy and in the extract prepared with methanol for blue poppy (Buran et al., 2022). In this study, it was determined that the TPC in the tea leaves extracts prepared in 20% and 50% ethanol were higher than extracts prepared in water. The TPC in tea leaf extract obtained from boric acid-treated soils was found to be higher only in the B1 and B3 groups in ethanol than in water. No difference was observed in terms of TPC in the extracts of tea leaves obtained from group B5 prepared in solvents such as water and ethanol. It can be said that solvent and boric acid affect the increase of TPC for the tea plant. On the other hand, high boric acid application to the soil may negatively affect the TPC of the tea plant.

Yolci et al. (2022) investigated the effect of dissolving the flowers of the plant called safflower or false saffron (*Carthamus tinctorius* L.) in different solvents such as water, methanol, acetone, and ethanol on the total concentrations of phenolic and flavonoid substances, and total antioxidant activity changes in flowers. For safflower flowers, water was reported to be the most suitable solvent for the highest TPC and antioxidant activity, while methanol was the most suitable solvent for TFC. In another study, extraction solvents were tested for six plant species grown in Sudan and the findings showed that ethanolic (50% ethanol, and 70% ethanol) and acetone extracts were rich in phenolic and flavonoid compounds and thus may contribute to high antioxidant activity (Dirar et al., 2019). In this study, when the TFC and AC of tea extracts brewed in water, 20% and 50% ethanol were compared, it was determined that the extracts brewed in ethanol had higher flavonoid content and AC than water. Thus, it can be said that changing the solvent of tea, which is consumed with water in daily life, as in other plants, can improve its quality.

As a result, TPC, TFC, and antioxidant activity in the green tea extracts may vary depending on the solvents they are prepared. It was detected that especially ethanol is a better solvent for the tea plant than water. According to that boric acid application can improve the green tea quality provided that it is at certain doses, while high doses may hurt the contrary.

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Research article

Investigation of bisphenol A (BPA) effects on germination and development of wheat and chickpea

Kurtulus Adiguzel[1], Mehmet Emre Erez*[1]

[1] Van Yuzuncu Yil University, Faculty of Science, Department of Molecular Biology and Genetics, 65100, Van, Türkiye

Abstract

Bisphenol A (BPA) is a chemical compound used in the production of various plastics. Its effects on health have been the subject of publications and scientific debate. The current study was carried out to reveal the effects of bisphenol A at various concentrations on seed germination and seedling development of wheat and chickpea plants. At the first step, seeds of wheat and chickpea were planted in sterile petri dishes and imbibed with 0, 1, 5, 10, 20, 40 and 50 mg L^{-1} Bisphenol A concentrations. Germination percentage, vigor index, radicle length, and plumula length were calculated. In the second step, the seeds were first germinated in sterile petri dishes, and seedlings were exposed to the same BPA concentrations. In addition to seedling development measurements, chlorophyll, carotenoid contents, and phenolic and flavonoid changes were analyzed. Stomatal aperture status in wheat seedlings was also monitored. The effect of BPA concentrations varied greatly depending on the plant species. Likewise, their effects on germination and development stages are highly variable. Root and stem lengths decreased due to increasing BPA concentrations. Regarding the effects of BPA on development, 40 and 50 mg L^{-1} concentration applications caused an increase in chlorophyll in wheat and a significant decrease in chickpea plants. Phenolic and flavonoid values showed differences depending on the application dose. It was noticed that their amounts increased significantly at concentrations higher than 20 mg L^{-1}. The cadmium toxicity effect varied depending on the seed species and cadmium concentration. While 1 and 5 mg L^{-1} applications did not cause a negative effect on germination and development, it caused inhibitory effects at high concentrations. BPA concentration in nature is increasing day by day. These findings provide invaluable information on the underlying effects and concentration limit of BPA on crop growth.

Keywords: *Bisphenol A; endocrine disruptors; germination; seedling; toxicity*

1. Introduction

Bisphenol A [2,2-bis (4-hydroxyphenyl) propane] is a widely used chemical compound that was first synthesized by Russian chemist Alexander Dianin in 1891 and is one of the highest-volume chemicals produced worldwide since the 1950s. BPA has been reported to be used primarily to produce polymer materials such as epoxy resins, polycarbonate plastic, and polysulfone resins. For this reason, its presence has been found in all environments, including the hydrosphere, lithosphere, and even the atmosphere (Tsai, 2006). The use of BPA in polycarbonate and epoxy resins results in the release of BPA into the environment through wastewater treatment organism effluents (Melcer and Klečka, 2011). Endocrine-disrupting chemicals, such as bisphenol, have the potential to disrupt human, animal, and plant normalcy. These substances can enter plants through their roots and the surrounding air, where they can impede the function of certain hormones and enzymes. Numerous investigations revealed that these chemicals have a detrimental impact on a variety of plant physiological functions, including germination, photosynthesis, etc. (Saraswat et al., 2024).

* Corresponding author.
E-mail address: emreerez@hotmail.com (M. E. Erez).
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Worldwide production of BPA is constantly increasing and will reach 6.2 million tons (MT) in 2020. According to estimates, the amount of BPA is expected to reach 7.1 MT by 2027 (Abraham and Chakraborty, 2020). Although bisphenol and its derivatives have been detected in all environments, many studies have been carried out on different organisms related to BPA. Bisphenol types have good stability, permanence, and bioaccumulation and show similar structure and properties (Chen et al., 2016). Increasing exposure time to BPA causes the formation of brown spots on wilted leaves in *Arabidopsis thaliana* and broad beans; it has also been reported that it causes a decrease in the number of leaves and aggravation of local necrosis in lettuce (Ferrara et al., 2006; Qiu et al., 2013). Also, tomatoes accumulate large amounts of BPA in their roots and transfer it to their branches, but not all BPA undergo biotransformation and detoxification, so its toxic effect continues. (Ferrara et al., 2006).

BPA and its derivatives harm not only human health but also the soil's microbiological and biochemical equilibrium, plant growth, and plant development (Zaborowska et al., 2023). BPA is quickly absorbed by plants from water through their roots, where it is then converted to several glycosidic molecules. Plants glycosylate BPA to increase the parent compound's estrogenic properties. Peroxidase and polyphenol oxidase are two oxidative enzymes that are intimately linked to the metabolism of BPA. According to a study on rice plants, BPA can be absorbed from the roots of seedlings quickly and transferred to their leaves (Noureddin et al., 2004). In another study it was reported that BPA sorption has a high potential to cause damage to the entire plant (Ferrara et al., 2006).

BPA has concentration-dependent effects on plant germination and development, with both low and high quantities having either promoting or inhibitory effects (Pan et al., 2013). The effects of BPA on plants are generally concentrated in its effects on enzymes, mineral uptake, pollen tube formation, microorganisms, and nucleic acids. The known effects of BPA in plants are increased respiratory enzyme activity and mineral element absorption in roots (Nie et al., 2015), changes in chlorophyll synthesis and effects on stomatal opening (Jiao et al., 2017), cell division and growth (Adamakis et al., 2013), increased levels of reactive oxygen species (ROS), lipid peroxidation, and suppression of root and seedling elongation (Pan et al., 2013; Wang et al., 2015; Zhang et al., 2016). According to the literature, plants detoxify BPA through a variety of metabolic pathways, and exposure to BPA triggers the signaling of ROS (Babu et al., 2013; Tian et al., 2014).

Germination is an important step, as it is the starting point of plant life and is affected by all environmental conditions and chemicals. Among organisms, plants are one of the most affected by environmental effects due to their sessile characteristics (Eskin et al., 2013). Increasing use of BPA in the developing world will increase the number of living organisms exposed to it. The effects of pesticides, herbicides, or similar chemicals should be evaluated in terms of human and environmental health.

Wheat and chickpea plants are among the most highly productive and widely cultivated crops in the world, so the study presented aims to determine the effects of different BPA concentrations on germination and seedling growth.

2. Materials and methods

2.1. Seed selection and BPA solutions

Wheat (*Triticum vulgare* L. Doğu-88 genotype) seeds used in the study were obtained from Van Yuzuncu Yil University, Faculty of Agriculture, and chickpea (*Cicer arietinum* L.) seeds were obtained commercially. BPA (Sigma-Aldrich) in crystal form was preferred for our study. First, a stock solution was prepared at a concentration of 50 mg L^{-1}, and other concentrations (1, 5, 10, 20 and 40) were diluted from the stock solution. 80 µl of absolute ethanol (Merck) was used to completely dissolve BPA. The same amount of ethanol was added to the internal control group.

2.2. Germination assay

Sterile glass petri dishes were used for the germination application. Two layers of sterile blotting paper were placed in the petri dishes. Unharmed, uniform 10 wheat and 6 chickpea seeds were placed separately in each petri dish. BPA solutions were added at application concentrations (1, 5, 10, 20, 40 and 50 mg L^{-1}) to each of the Petri dishes containing the seeds. BPA solutions were used: 6 mL for wheat and the last 10 mL for chickpeas. Ethanol-containing water was used for the internal control group, and pure water was used for the external control group. All petri dishes were covered with parafilm to prevent solution evaporation. The germination study was started under controlled conditions (24°C temperature, 65% humidity, 8/16 photoperiod).

2.2.1. Germination percentage and vigor index

To evaluate the effects of BPA on seed germination; germination percentage, inhibition/stimulation status, and vigor index values were calculated according to the formulas:

Germination percentage:

$$Germination\ \% = \frac{Germinated\ Seede}{Total\ seed} * 100$$

Stimuli/inhibition:

$$BPA\,\frac{inhibition}{stimulation}\,\% = \frac{Germiantion\ of\ Sample - \text{Control}}{Control} * 100$$

(-indicate inhibition, + stimulation)

BPA seed vigor index = Germination percentage * total length

2.3. Seedling assay

For the seedling effect of BPA, wheat and chickpea seeds were pre-germinated in sterile petri dishes. The germinated seeds were transferred to jars containing two layers of sterile blotting paper. Hoagland medium was added to the jars to continue the development phase (Hoagland and Arnon, 1950). For each 50 mL of Hoagland's solution, 2.42 mg Iron (III) chloride (Merck) and 75 mg EDTA (Sigma-Aldrich) were added. The germinated seeds were allowed to acclimate for one day. At the end of the period, different concentrations (0, 1, 5, 10, 20, 40 and 50 mg L^{-1}) of BPA were applied to the seedlings. Glass jars were sealed with parafilm. Seedling development was observed for seven days in appropriate light and temperature (24°C temperature, 65% humidity, 10/14 light period).

2.3.1. Determination of photosynthetic pigment contents

Plant materials crushed in liquid nitrogen were transferred to 80% acetone (Merck) and homogenized in a Sonicator (Wiggen Hauser) for 1 minute. The samples were centrifuged at 4000 rpm and the supernatants were transferred to clean falcon tubes. 2 mL of 80% acetone was added and readings were performed on the spectrophotometer (Shimadzu). Pigment content measurements were made according to Arnon (1949). The amounts of photosynthetic pigments were calculated in mg g-L according to the formulas:

Chlorophyll-a = [A663x12.70 - A645x2.49] [(V/1000)*W]

Chlorophyll-b = [A645x22.90 - A663x4.68] [(V/1000)*W]

Total chlorophyll = [A645x20.2 + A663 x 8.02] [(V/1000)*W]

Carotenoid = [A480 + A663x0.114 - A645 x 0.638/112.50] [(V/100)*W]

2.3.2. Total phenolic and flavonoid contents

Total phenolic contents were measured using the Folin-Ciocalteu reagent (Sigma-Aldrich) (Dalar et al., 2012). 25 µL of plant extract was mixed with 125 µL of Folin-Ciocalteu (1: 10, v/v) reagent and shaken for 3 minutes. 125 µL, 6% Na_2CO_3 (Merck) was added, and the microplate was shaken for 10 minutes. At the end of the period, absorbance values were measured at 600 nm. Results were expressed as mg gallic acid (GA) equivalent/g according to the gallic acid standard (Merck) curve (Dalar and Konczak, 2013).

For total flavonoid values, 25 µL plant extract was mixed with 125 µL ultra-distilled water and 7.5 µL 5% $NaNO_2$ (Merck) (1:20 w/ v). After incubation for 5 minutes, 15 µL of 10% $AlCl_3$ $6H_2O$ (Sigma-Aldrich) was added. Samples were incubated at room temperature for 6 min, then 50 µL of 1 M NaOH was added and mixed thoroughly until pinkish color appeared. Results were expressed as mg Rutin (R) E/g according to the rutin standard curve. Analyses were performed in three replicates (Akbay et al., 2003).

2.4. Statistical analysis

Experiments were carried out via three replications. The average value ± standard deviation was used to present the results. Using Minitab software, the acquired findings were analyzed using one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$.

3. Results

3.1. Effect of BPA on germination

BPA application caused variable responses in the germination of wheat and chickpea seeds. The responses exhibited different effects depending on the plant species and concentration. While germination inhibition in wheat seeds started at a concentration of 20 mg L^{-1}, in chickpea seeds it started at a concentration of 10 mg L^{-1}. Although it caused a slight inhibition in chickpea seeds at 5 mg L^{-1}, the seeds were not affected by BPA concentrations of 1 and 5 mg L^{-1} (Fig. 1A-B).

When the inhibition percentages of BPA concentrations were examined, it was determined that there was a significant difference between chickpea and wheat seeds. In wheat seeds, 4% inhibition was detected at 20 mg L^{-1}, 5% at 40 mg L^{-1}, and 9% inhibition at 50 mg L^{-1} concentration. In chickpea seeds, 11% inhibition was observed at 20 mg L^{-1}, 28% at 40 mg L^{-1}, and 33% inhibition at 50 mg L^{-1} concentration.

Among the BPA applications, the highest inhibition value was observed in chickpea seeds at a concentration of 50 mg L^{-1}. Interestingly, 50 mg L^{-1} application in wheat seeds caused 9% inhibition, while more than 3 times inhibition was detected in chickpea seeds at the same concentration (Fig. 1C-D).

While there was no significant difference in germination inhibition between 20 and 40 mg L^{-1} concentrations in wheat seeds, a significant inhibition difference was detected between both applications in chickpea seeds.

Similar effects were seen in vigor index values depending on the germination rate. While the 10 mg L^{-1} concentration in wheat seeds did not cause inhibition, it caused a significant decrease in the vigor index value. It was determined that the vigor index level decreased due to the germination inhibition observed in chickpea seeds at a concentration of 5 mg L^{-1}. Interestingly, 1 mg L^{-1} BPA application caused to be higher than the control group for the vigor index values in both wheat and chickpea seeds. In addition, even though increasing BPA concentration in chickpeas reduced the germination percentage, no significant difference could be detected between the vigor indices of 40 and 50 mg L^{-1} applications (Fig. 2A-B).

3.2. Effect of BPA on seedling

3.2.1. Effect on root elongation

When the effects of BPA on root lengths in wheat seedlings were examined, it was determined that 1, 5, and 10 mg L^{-1} BPA applications stimulated root development at a much higher rate compared to the control group, and there was a significant difference, especially between the 1 mg L^{-1} application and the control group. It was observed that 40 and 50 mg L^{-1} applications had a negative effect on root development.

The application of 1 mg L^{-1} concentration of Bisphenol A in the root length of chickpea seedlings increased the root length significantly, but no significant difference was observed in all other concentration groups. It was determined that all BPA applications except 20 mg L^{-1} caused the root length to be longer than the control (Fig. 3 A-B)

3.2.2. Effects on pigment concentration

In terms of chlorophyll values, it was determined that BPA affected chickpea seedlings more than wheat seedlings. In all applications of BPA, chlorophyll values were lower than the control. An increase in chlorophyll values was detected in 10 and 20 mg L^{-1} applications in chickpea seedlings. While there was no significant difference between control and application values in wheat seedlings, it was calculated that the chlorophyll values of the 20 mg L^{-1} BPA application had the highest value (Fig. 4A).

Carotenoid values were similar to chlorophyll values. Unlike the chlorophyll ratios, no decrease was observed in chickpea seedlings at 1 and 5 mg L^{-1} concentrations. Again, the

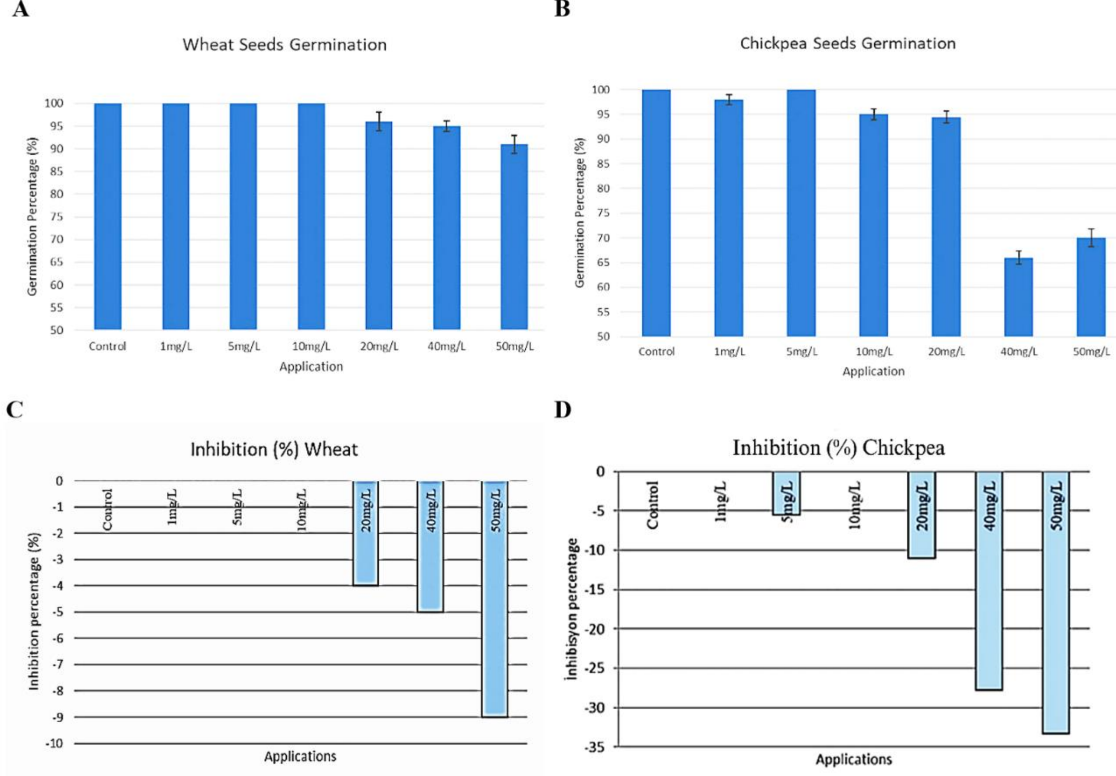

Fig 1. Effects of BPA on seed germination (A) Wheat seeds germination percentage, (B) Chickpea seeds germination percentage, (C) Wheat seeds germination inhibition, (D) Chickpea seeds germination inhibition.

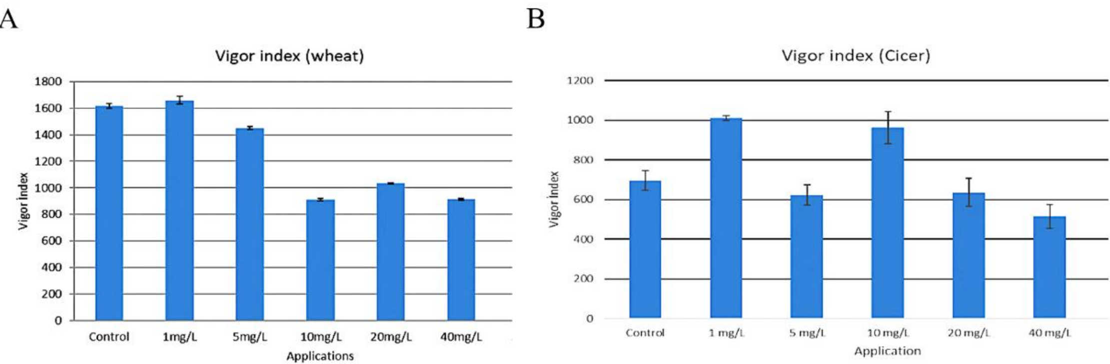

Fig 2. The effects of BPA applications on vigor index (A) wheat, (B) chickpea.

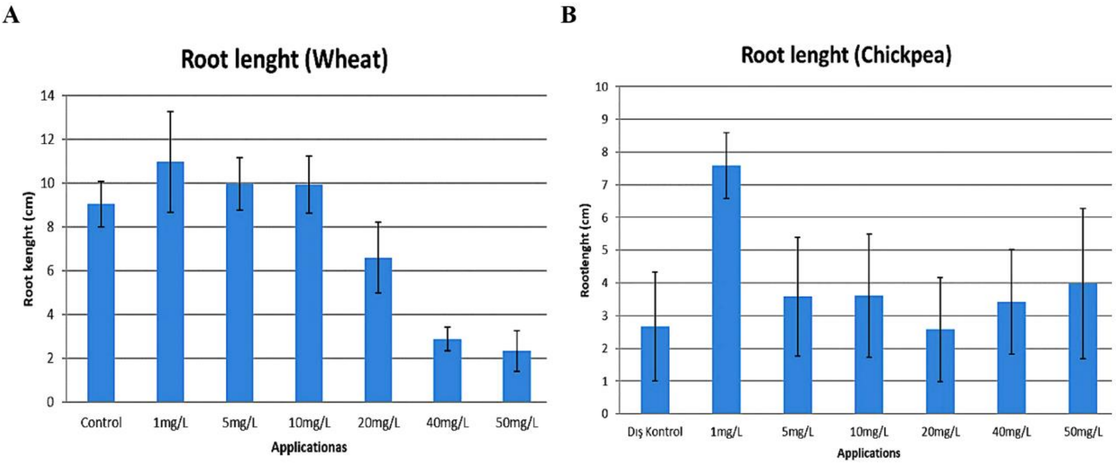

Fig 3. The effects of BPA applications on root length of seedlings (A) wheat, (B) chickpea.

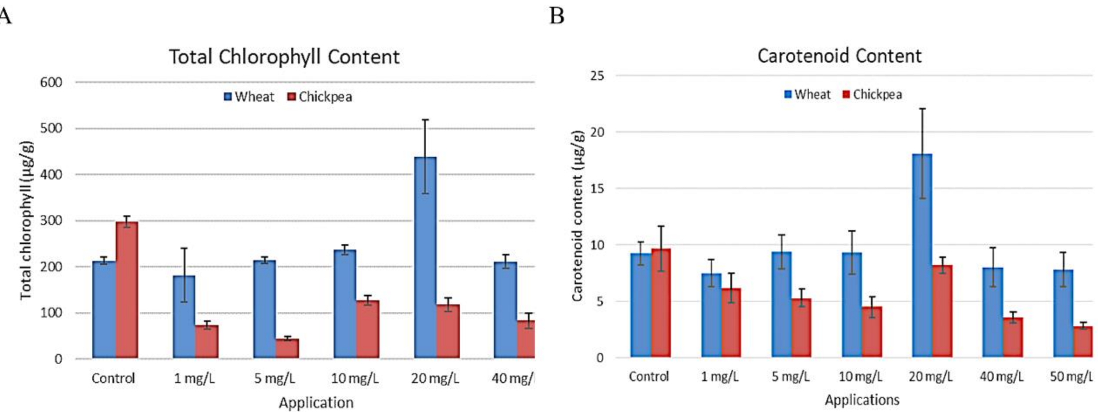

Fig 4. The effects of BPA applications on pigment content (A) total chlorophyll, (B) total carotenoid.

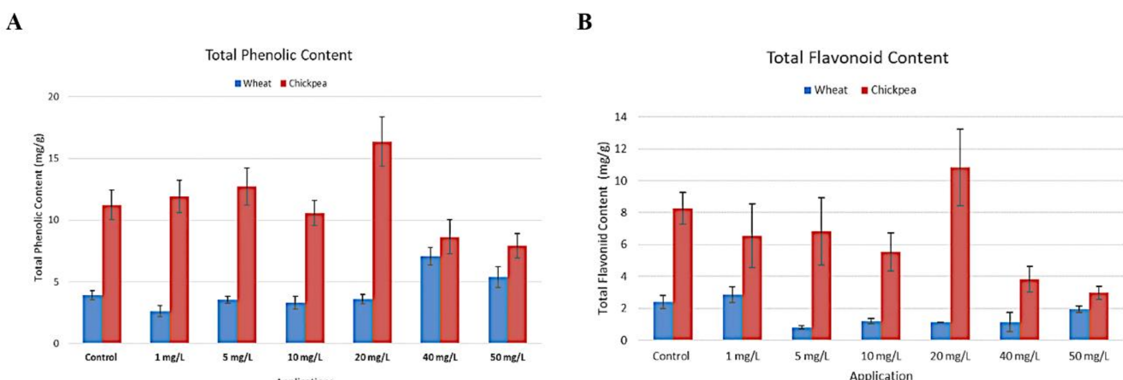

Fig 5. The effects of BPA applications (A) total phenolic content and (B) total flavonoid content.

highest carotenoid values for both wheat and chickpea seedlings were recorded in the 20 mg L^{-1} application (Fig. 4B).

3.2.3. Effects on phenolic and flavonoid content

Wheat and chickpea seedlings exhibit different reactions in their phenolic contents. While a significant increase was observed in wheat seedlings up to 20 mg L^{-1} application, significant decreases were detected at 40 mg and 50 mg L^{-1} applications. However, while no significant change was observed in chickpea seedlings until 20 mg L^{-1} application, significant increases were observed in 40 and 50 mg L^{-1} applications. The highest phenolic substance content was calculated when 20 mg L^{-1} was applied to wheat plants and 40 mg L^{-1} was applied to chickpea seedlings. (Fig. 5A).

Total flavonoid content in wheat seedlings decreased steadily with the increment of concentration, but in the 20 mg L^{-1} application, the flavonoid value was found to be higher than all applications, including the control. While decreases were observed in chickpea seedlings with 1-5 and 10 mg L^{-1} applications, no significant change was observed between other applications. (Fig. 5B).

3.2.4. Stomatal aperture effect on wheat leaves

Stomatal apertures in wheat plant leaves were determined by electron microscopy. Stoma analysis could not be performed on chickpea leaves because they were very poorly developed and had a gold coating problem depending on the leaf form.

In the electron microscopy analysis, it was observed that stomatal aperture was markedly open in the control, 1 mg L^{-1} and 5 mg L^{-1} concentrations of wheat leaves. In other applications, it was observed that the stomata were completely closed, especially in the application of 40 mg L^{-1} BPA (Fig. 6).

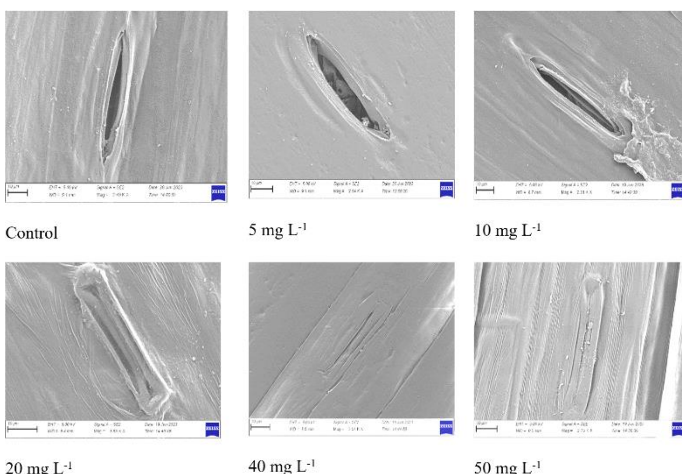

Fig 6. Stress-related stoma images of wheat by electron microscopy.

4. Discussion

Penetration of a radicle from the seed coat and growth into a new plant, known as seed germination marks the beginning of plant life. The germination period is the stage when plants are most sensitive. Therefore, seed germination is one of the established criteria often used to screen plant species for their tolerance to toxicity. According to earlier research, different BPA doses can have varying impacts on the germination of

seeds. BPA has concentration-dependent impacts on plant growth and performance, with low and high BPA concentrations observed to have promotion and inhibitory effects, respectively (Xiao et al., 2020). In our study, different concentrations of BPA caused variable effects on the germination of wheat and chickpea seeds. This may depend on many factors, such as the cotyledon status of the plants, the seed coat, and their genetic potential.

In the current study, it was determined that low doses of BPA did not inhibit germination, while high doses inhibited it. Recent study results revealed that BPA may have a stimulating effect at low concentrations and an inhibitory effect at high concentrations (Pan et al., 2013; Qiu et al., 2013). A BPA concentration of > 50 mg L^{-1} was found to inhibit the germination of seeds of *Arabidopsis thaliana* L. and *Cicer arietinum* L. (Dogan et al., 2010; Tian et al., 2014). This may be connected to greater BPA dosages inhibiting energy metabolism during seed germination (Xiao et al., 2020). In addition, it can be thought that BPA has a similar biological activity to gibberellic acid in low doses and shows a behavior that mimics this hormone. The use of multibiomarkers showed that a reduction in the plant hormone GA_3 is closely linked to the suppression of root growth. The amount of GA_3 was likewise reduced by BPA at the lowest concentration. Following a reduction in GA_3 and inhibition of root growth, H_2O_2 and O_2 generation as well as the activation of antioxidant defenses (Vujčić et al., 2023). Several possibilities have been suggested for BPA to affect seed germination. The first of these is that it has been reported that BPA activates the *PF3* gene of phytochromes with a helix-loop-helix transcription feature in seeds exposed to light during germination. Accordingly, it is suggested that seeds exposed to light generally have higher amounts of phytochrome A. The possible effect of BPA at high concentrations on phytochromes is thought to cause germination inhibition (Hanumappa et al., 1999; Pan et al., 2013).

Low-dose BPA stimulates stem cell elongation and proliferation and has a cytokinin-like effect, which promotes root growth (Terouchi et al., 2004). Also, low-dose BPA exposure promotes stem cell elongation and division, raising the amounts of the hormones gibberellin (GA), zeatin (ZT), and indole-3-acetic acid (IAA) in roots and controlling the growth of primary and lateral roots (Li et al., 2017). While decreases were observed in chlorophyll values in the study, a significant increase was observed, especially at the 20 mg L^{-1} dose. Interestingly, a similar situation was observed in the phenolic and flavonoid contents. This data indicates that the equivalent value of BPA in the seedlings is 20 mg L^{-1} and that the seedlings exhibit the highest response at this concentration. At other high doses (40 and 50 mg L^{-1}), it is seen that the seedlings are damaged and cause metabolic deterioration. BPA in high doses is related to the ROS (reactive oxygen species) effect. Exposure to high doses of BPA can lead to the accumulation of ROS (Ferrara et al., 2006; Tian et al., 2014). The thylakoid membrane and chloroplast structure are oxidized and damaged by the excess ROS, which also lowers the amount of chlorophyll and the efficiency with which light energy is absorbed and converted. These effects all have an impact on photosynthesis (Li et al., 2018). This situation also directly affects the stoma aperture. The stomatal aperture in wheat leaves is quite small at doses of 40 and 50 mg L^{-1}. In a study, it was stated that the possible mechanisms by which carotenoids play a role in reducing BPA stress in tobacco are related. It provided a new strategy to increase the phytoremediation efficiency of plants in BPA-contaminated soil (Fu et al., 2023). Growth indicators and chlorophyll content can be impacted by BPA in a dose-related way. When BPA is present in low concentrations (2 mg L^{-1}), it stimulates the growth of pea seedlings. However, when BPA is present in higher concentrations, it significantly inhibits the growth of pea seedlings. This is because the reduced chlorophyll content causes a decrease in photosynthesis (Siddqiui et al., 2022).

Research has indicated that when plants are exposed to BPA concentrations below 3.0 mg L^{-1}, their development is enhanced. These effects were associated with increased respiratory enzyme activity and mineral element absorption in roots (Ali et al., 2016; Xiao et al., 2019), chlorophyll synthesis (Jiao et al., 2015), and stomatal opening (Jiao et al., 2017). Additionally, improved photosynthetic system II (PS II) efficiency was linked to these effects (Zhang et al., 2015). However, more than 3.0 mg L^{-1} BPA exposure exhibited negative impacts on plant growth, as demonstrated by elevated ROS and lipid peroxidation levels as well as suppression of root and seedling elongation (Pan et al., 2013).

The current study and literature research present that BPA has different toxic effects not only on humans and animals but also on plants. Despite the increasing use of BPA, studies with plants are important for raising the awareness of future generations.

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Research article

Changes in stemness properties of human adenoid-derived mesenchymal stem cells during *in vitro* aging

Melek Yuce*[1], Esra Albayrak[1], Gokhan Akgul[2], Nurcihan Yagci[3], Esra Tekcan[4]

[1] *Ondokuz Mayis University, Stem Cell Research & Application Center, 55270, Samsun, Türkiye*
[2] *Samsun Education and Research Hospital, Department of Otorhinolaryngology, 55090, Samsun, Türkiye*
[3] *Istanbul University, Nanotechnology and Biotechnology Institute, 34320, Istanbul, Türkiye*
[4] *Ondokuz Mayis University, Faculty of Medicine, Central Laboratory, 55270, Samsun, Türkiye*

Abstract

Mesenchymal stem cells (MSCs) have significant therapeutic potential in gene therapy. *In vitro* replicative senescence causes a decrease in the proliferation capacity of MSCs and changes in stem cell properties. In this study, adenoid tissue was focused as a new MSC source. The stem cell properties and the proliferation potential of adenoid-derived MSCs after the long-term *in vitro* replicative senescence were investigated. Adenoid-derived MSCs (A-MSCs) were cultured up to passage 20 and were analysed for cell morphology, proliferative capacity, differentiation potential, and surface marker expression. In addition, the expression profile of cell cycle, apoptosis, and senescence-related genes were evaluated. After *in vitro* replicative senescence, A-MSCs did not show any significant morphological differences. The proliferation potential of A-MSCs was rapid up to passage 16, and a reduction in the proliferation potential of senescent cells *in vitro* was observed depending on the passage number. The differentiation potential of late-passage A-MSCs was also reduced compared to early-passage cells. A-MSCs also provided significant closure at the 8th hour in early passages in terms of closure of the scratch area, while late passage A-MSCs exhibited a similar closure profile at the 24th hour. At the transcriptional level, the upregulation of the *BAX* gene and the downregulation of the *p21* and *p53* genes suggest that late-passage A-MSCs may not exhibit a senescence profile. In conclusion, A-MSCs have significant potential for clinical use due to the sustainability of MSC properties and their ability to proliferate and migrate with long-term culture.

Keywords: *Adenoid; cell proliferation; characterization; mesenchymal stem cells; senescence*

1. Introduction

Mesenchymal stem cells (MSCs) are adult multipotent cells that can self-renew and differentiate into non-mesodermal origins such as mesodermal and neuronal cells, cardiomyocytes, hepatocytes, or epithelial cells, particularly in fat, bone, and cartilage tissue (Noronha-Matos and Correia-de-Sá, 2016; Hmadcha et al., 2020). After the first identification in the bone marrow (Friedenstein et al., 1976), mesenchymal stem cells derived from many tissues such as adipose tissue (Mazini et al., 2019), muscle (Camernik et al., 2019), peripheral blood (Wu et al., 2015), hair follicles (Wang et al., 2020), teeth (Ledesma-Martinez et al., 2016), placenta (Deng et al., 2024), umbilical cord (Rajput et al., 2024), cord blood (Chang et al., 2009), lung (Hoffman et al., 2011), heart (Hoogduijn et al., 2007), endometrium (Bagheri-Mohammadi et al., 2019) are shown to be promising sources in terms of proliferation and differentiation potential into different cell types.

MSCs exhibit significant potential for tissue repair and gene therapy. Their ability to divide symmetrically and asymmetrically gives these cells self-renewal and versatile differentiation capability, thus making them promising

* Corresponding author.
E-mail address: melek.yuce@omu.edu.tr (M. Yuce).
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candidates for regenerative medicine. (Bonab et al., 2006). Furthermore, their immunomodulatory properties make them advantageous for autologous applications in addition to allogeneic applications (Choudhery et al., 2014). To date, bone marrow MSCs (BM-MSCs) are the best-characterized stem cells widely used in experimental and clinical studies. However, the collection of BMs requires a challenging and invasive process. However, the low number of obtained cells and decreased proliferation ability and differentiation potential due to age led the researchers to search the alternative sources (Kern et al., 2006; Choudhery et al., 2014).

There is an imperative to culture and propagate MSCs *in vitro* before their therapeutic usage in the clinic. Therefore, it is important to determine the effect of *in vitro* aging on cellular properties before applications of MSC-based therapeutic strategies (Donega et al., 2014). The decrease in the proliferative abilities of MSCs, the change in their morphology, differentiation potential, and aging-related gene expression with long-term *in vitro* culture of MSCs have been demonstrated to cause many problems before the clinical use of these cells (Gu et al., 2016). Determination of alternative MSC sources is very important for the development of future cell therapies and stem cell banking due to the difficulty of sample collection, the low number of stem cells, and the decrease in proliferation and differentiation potentials depending on donor age (Feng et al., 2020). Nowadays, preserving distinct stem cell types and making them ready for treatment at the right time have indisputable importance. This seems possible with stem cell biobanks, but there are still significant shortcomings in providing adequate tissue and stem cells. Umbilical cord blood comes to the fore in stem cell banking because it is an easily accessible waste tissue. In addition, new born tissues such as umbilical cord tissue, placenta tissue, amniotic fluid, and amniotic membrane have become popular in the last few years for stem cell sources and banking. However, waste tissues such as adenoid tissue, tonsil tissue, and foreskin also have important potential as stem cell sources for biobanks (Li et al., 2023; Zhu et al., 2024). The importance of the stromal cells involved in secondary lymphoid organs (SLOs), which is an important part of the immune system in defence against invading pathogens, has been highly investigated (Mueller and Germain, 2009; Genovese and Brendolan, 2016). Tonsil-derived MSCs (T-MSCs) obtained from tonsil, one of the SLOs, have recently attracted intense interest as a source of MSCs due to their easy availability as waste tissue after tonsillectomy, their relatively high proliferation rate, and low allogenicity (Ryu et al., 2012; Shin et al., 2018; Cho et al., 2019; Oh et al., 2019). However, insufficient data are available on whether adenoid tissue can be used as a source to isolate multipotent MSCs or not. Adenoid tissue, which is commonly found in childhood and can be easily accessed as waste tissue, has the potential to be an important MSC source. In addition to their capacity to differentiate into adipogenic, osteogenic, and chondrogenic lineages, recent studies have revealed that they can differentiate into immature olfactory sensory neurons *in vitro* (Guo et al., 2023). It is important to investigate what extent they preserve their *in vitro* replicative potential and stem cell properties considering the regenerative potential of MSCs and their importance in cellular therapy. Therefore, in this study, we examined changes in cell morphology, proliferation rate, cell surface antigen expressions, migration ability, differentiation ability and many cellular process-related gene expressions of A-MSCs after replicative senescence *in vitro*.

2. Materials and methods

2.1. Adenoid-derived mesenchymal stem cell isolation and culture

A-MSCs were isolated from adenoid tissue taken during an adenoidectomy operation in the ear and nose and throat diseases department of Samsun Training and Research Hospital under the patient's consent. This study was approved by Ondokuz Mayis University Clinic Research Ethics Committee (Decision number: 2021/352). The adenoid tissues were washed twice with phosphate-buffered saline (PBS) and then incubated in 10 ml PBS supplemented with 10% antibiotic/antimycotic for 15-20 min at room temperature (RT). A-MSC isolation was performed according to the procedure described in the previous publication by Yuce and Albayrak, (2022). Briefly, tissues were divided into small pieces of 1-3 mm^3 in size. They were then enzymatically digested with Dulbecco's modified Eagle's medium (DMEM) supplemented with collagenase type-I at 37°C for 30 min and then filtered through a 100 µm cell strainer. Cells were incubated with DMEM (Sigma) containing 10% FBS, and 1% antibiotic/antimycotic (Sigma Aldrich).

2.2. Immunophenotyping on adenoid-derived mesenchymal stem cells

To evaluate the mesenchymal cell characteristics of the newly isolated adenoid-derived cells with replicative senescence, we performed the immunophenotyping assay. The cells at different passage numbers (p:4, p:12, and p:20) were collected from the culture flask by using 0.25% trypsin. Then, the cells were labelled with fluorescein Isothiocyanate (FITC)-conjugated anti-human CD90, allophycocyanin (APC)-conjugated anti-human CD73, FITC-conjugated anti-human CD44, phycoerythrin (PE)-conjugated anti-human CD34, allophycocyanin (APC)/Cyanine7-conjugated anti-human CD45 monoclonal antibodies at 40°C for 30 min. After the incubation, the labelled cells were analysed by flow cytometry (n=3) (Cytoflex S, Beckman Coulter).

2.3. Determination of multi-lineage potential of A-MSCs

Adipogenic and osteogenic differentiation protocols were performed for A-MSCs at passages 4 and 20. The protocol was performed according to Yuce and Albayrak (2022). A-MSCs at a density of $1x10^5$ were seeded in six-well culture dishes exposed to a differentiation medium for three weeks and renewed every 2 days. At the end of the 3 weeks when cell differentiation was observed, cells were washed with PBS. They were then fixed with 4% paraformaldehyde (PFA) for 30 minutes. After fixation, cells were stained with 2% Oil red O solution (Sigma Aldrich) for 1 hour at room temperature for adipogenic differentiation and 2% Alizarin Red S solution (Sigma Aldrich) for 15 minutes for osteogenic differentiation. After washing with PBS, the cells were observed under inverted microscopy. The experiments were performed in triplicate.

2.4. Determination of growth rate and doubling time

To indicate the effect of replicative senescence on the growth rate and doubling time of A-MSCs, we determined the growth rate and calculated the doubling time. The cells in passages 4, 8, 12, 16, and 20 were used for the determination of

the growth curve graph. The cells at passages 5 and 50 were used for the calculation of doubling time. For this, A-MSCs were seeded in a 12-well plate at a density of $3x10^4$ cells and incubated for 7 days (n=3). The cells were trypsinized and counted following trypan blue (Sigma Aldrich) staining every day post-seeding for determination of growth rate. Besides, population doubling time (PDT) and the number of cell population doubling (NCPD) were calculated with the formulas given below (Yang et al., 2018).

$$NCPD=3.33\times\log(Nt/Ni)$$
$$PDT=(t-ti)\times\log\{2\times[\log(Nt/Ni)]-1\}$$

2.5. Cell proliferation assay

We performed MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay to exhibit the changes in the proliferation ability of A-MSCs with replicative senescence. We seeded the A-MSCs passage 5 and 20 onto 96 well-plate at a density of $2x10^3$ cells (n=3). The proliferation was measured on days 1, 3, 5, and 7. For measurement, MTT reagent was added to the cells at ten percent volume of the culture medium and the cells were incubated at 37°C for 4 hours. After the incubation, the dissolving solution (10% Sodium Dodecyl Sulphate (SDS) in 0.01 M Hydrochloric acid (HCL)) was added into the wells and incubated for 16 hours. The wells were measured by a microplate reader at 570 nm wavelength (Thermo Scientific™Multiskan™GOMicroplate Spectrophotometer) (Alcayaga-Miranda et al., 2015).

2.6. In vitro scratch assay

The migration abilities of A-MSCs post *in vitro* aging were indicated with *in vitro* scratch assay. $5x10^4$ cells at passages 7 and 20 were seeded onto 6 well-plate and the wound was created as a line with a 10 µl pipette tip onto the cells post reaching the confluence (n=3). The cells were washed with PBS to avoid the residues and were incubated in 2% FBS-DMEM at 37°C for 24 hours. The migration on the wound region was observed by phase-contrast microscope in time intervals (at 0, 8, 16, and 24 hours). The migration ability was quantified through the counting of the cells in the region by ImageJ analysis software (Alcayaga-Miranda et al., 2015).

2.7. Gene expression analysis post replicative senescence

The expression profiles of the aging-related genes on *in vitro* aged-adenoid-derived MSCs were analyzed by real-time polymerase chain reaction (RT-PCR). For that, total RNAs were isolated from the $5x10^4$ cells at passages 7 and 20 by NucleoZOL reagent (Macherey-Nagel). After RNA isolation, 5 µg RNA was converted to cDNA by cDNA synthesis kit (ProtoScript® II First Strand cDNA Synthesis Kit). Then, RT-PCR was performed with the interested primers listed in Table 1 by Maxima SYBR Green qPCR Master mix kit (Thermo Scientific). The gene expressions were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene and the Ct values were analysed by the 2-ΔΔCt method. The experiments were performed in triplicate.

2.8. Statistical analysis

The results are expressed as mean ± standard deviation. The statistical analysis was performed using GraphPad Prism software. The experiments were performed in triplicate and analysis of variance was used to analyse the variance among between groups. "2-tailed Student's t-test" was used to determine the significance level. The results were considered statistically significant if the values were $p < 0.05$.

3. Results

3.1. Morphological comparison of in vitro aged A-MSCs

The isolated cells from human adenoid tissue exhibited the fibroblast-like spindle-shaped morphology. We have observed that A-MSCs retained their characteristic spindle-shaped morp-

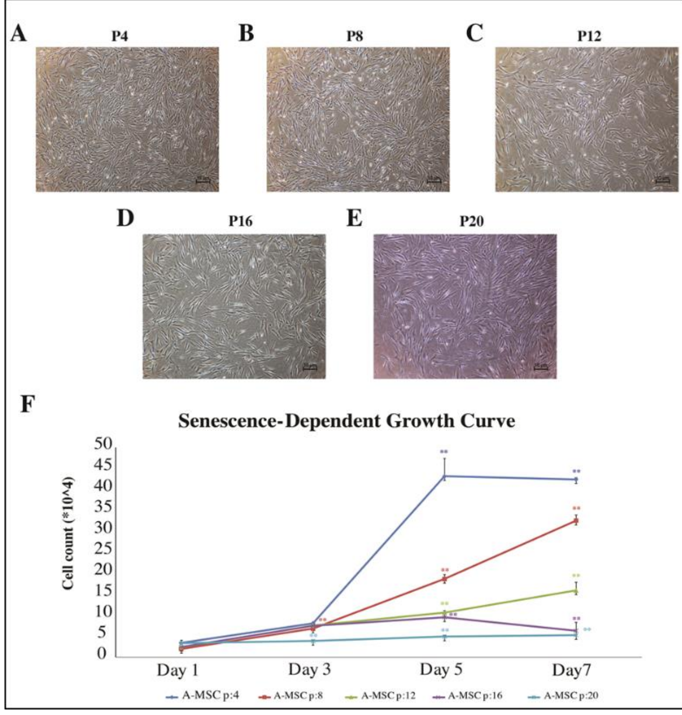

Fig. 1. Morphology of adenoid-mesenchymal stem cells depending on different passage number. The bright field microscopy views are presented for (A) passage 4, (B) passage 8, (C) passage 12, (D) passage 16, (E) passage 20 respectively (F) Senescence depending growth curve. The images are taken under 4X magnification. Scale bar: 10µm.

Table 1
The sequence information of the primers used for RT-PCR.

Gene	Forward primer	Revers primer
h*GAPDH*	5′-GTCTCCTCTGACTTCAACAGCG-3′	5′-ACCACCCTGTTGCTGTAGCCAA-3′
h*CCND1*	5′-TCTACACCGACAACTCCATCCG-3′	5′-TCTGGCATTTTGGAGAGGAAGTG-3′
h*CDKN1A*	5′-AGGTGGACCTGGAGACTCTCAG-3′	5′-TCCTCTTGGAGAAGATCAGCCG-3′
h*BAX*	5′-TCAGGATGCGTCCACCAAGAAG-3′	5′-TGTGTCCACGGCGGCAATCATC-3′
h*P53*	5′-CCTCAGCATCTTATCCGAGTGG-3′	5′-TGGATGGTGGTACAGTCAGAGC-3′
h*C-MYC*	5′-CCTGGTGCTCCATGAGGAGAC-3′	5′-CAGACTCTGACCTTTTGCCAGG-3′

hology until the 12th passage. A-MSCs at higher passage (p16, 20) exhibited a slightly more irregular morphology, although no major changes were observed (Fig. 1A-E). Therefore, we investigated the effect of *in vitro* aging on the growth of A-MSCs and generated growth curves for passages 4, 8, 12, 16, and 20. The decrease observed in the growth curves depending on the number of passages was greater in passages 16 and 20, defined as late passages. Because of the rapid increase in early passage 4 cells until day 5, 90-100% confluency was reached, so there was not significant change between the 5th and 7th day. Besides, we determined that the growth rate of A-MSCs at early passages (passages 4 and 8) was higher than the cells at late passage during *in vitro* aging (Fig. 1F).

3.2. The expressions of A-MSC surface antigens were maintained during in vitro aging.

The cell surface antigens are commonly used for identification of mesenchymal stem cells. The negative markers CD45, CD34, and positive markers CD73, CD90 are identified for mesenchymal stem cell characterization. In addition to them, CD44 expression is specifically important for the identification of A-MSCs. We analysed the expressions of these markers to investigate the effect of *in vitro* aging on A-MSCs. The plots of flow cytometry analysis are representatively shown in Fig. 2A. We compared passages 4, 12 and 20, and we found no significant changes in the ratio of CD34-, CD45-, CD73+, and CD90+ post-replicative senescence. However, although statistically significant change in CD44 ratio due to *in vitro* aging was observed, passages 4, 12, and 20 also showed over 97% positive expression (Fig. 2B). These findings reveal that the expressions of A-MSC surface antigens were maintained during *in vitro* aging.

3.3. The multi-lineage potential of A-MSCs during in vitro aging

Adipogenic and osteogenic differentiation capacities of A-MSCs have been investigated during *in vitro* aging. The findings obtained from the cells at passage 20 (late passage) were compared to the results obtained from the cells at passage 4 (early passage). To determine the adipogenic and osteogenic differentiation capacities of A-MSCs at passages 4 and 20, the cells were stained with Oil red O and Alizarin red, respectively following induction to differentiation with the special medium for 21 days. After the induction, we found that the lipid vesicles, which are the main indicator for adipogenic differentiation, were considerably decreased for passage 20 cells compared to passage 4 cells during *in vitro* replicative senescence (Fig. 3A). Similarly, the osteogenic indicator calcium deposits were decreased for passage 20 cells compared to passage 4 cells (Fig. 3B). These findings reveal that the *in vitro* aging suppressed the multi-lineage potential of A-MSCs.

3.4. In vitro aging caused the reduction of A-MSCs growth rate

MSC usage in clinic applications requires abundant cell numbers for efficiency. Thus *in vitro*, cell proliferation and population doubling time are very important parameters as much as cell sources and cell passage for cellular therapies. Therefore, we calculated the population doubling time and number of cell population doubling parameters. We found that the number of cell population doubling was significantly decreased around 2-fold in passage 20 A-MSCs compared to P:5 A-MSCs. In parallel with this result, the population doubling time was extended from 3.39 to 5.25 (Fig. 4A). These results indicated that the *in vitro* aging negatively regulated the doubling and growth rate of the A-MSCs.

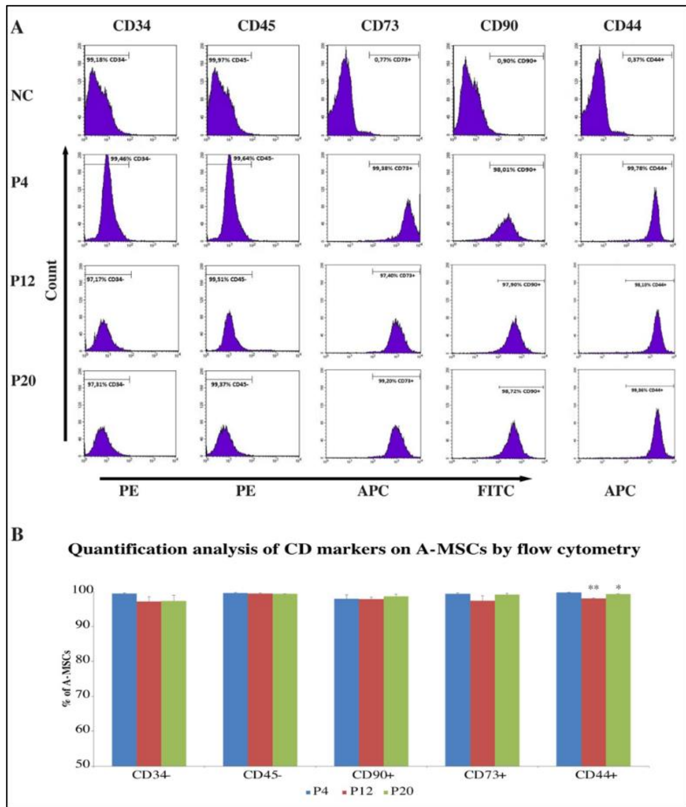

Fig. 2. Cell surface CD marker expression profile on A-MSCs at different passage number. The histogram plots (A) and quantification results (B) which belong to flow cytometry analysis of CD markers are presented. n=3, n=3, **p*<0,05, ***p*<0,01.

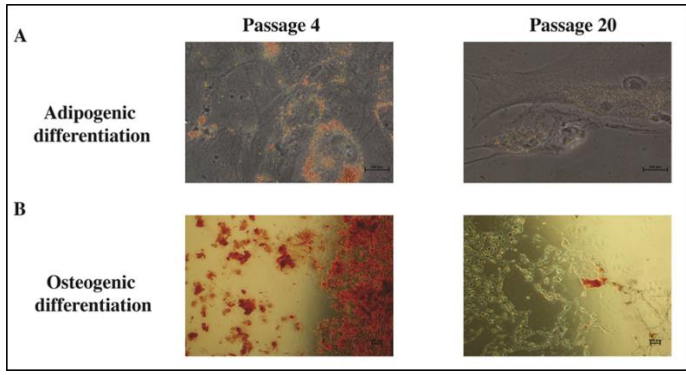

Fig. 3. Comparison of multilineage differentiation ability of adenoid-derived mesenchymal stem cells in different passages. (A) Adipogenic differentiation and (B) osteogenic differentiation abilities of adenoid-derived MSCs were evaluated by detection of oil vesicles and calcium deposition following Oil-Red-O and alizarin red staining on the cells at passage 4 and 20, respectively. Scale bar: 100μm.

In addition, we confirmed the proliferation capacity of A-MSCs with MTT assay during *in vitro* aging. We measured the proliferation in time intervals (day 1, 3, 5, and 7). We found that the absorbance values in passage 20 cells were significantly lower compared to passage five cells in the first 24 hours of culture. This reduction may be due to the low number of seeded cells. However, the proliferative potential of p20 cells was

relatively lower than that of p5 cells. We did not find a significant difference between day 3 and day 5, but a statistically significant decrease was observed on day 7 (Fig. 4B).

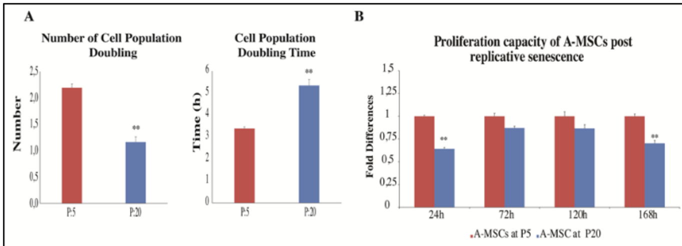

Fig. 4. Growth and proliferation rate of A-MSCs post replicative senescence. A) The number of cell population doubling (left) and the doubling time of the cell population (right) graphs are shown for A-MSCs post replicative senescence. B) The proliferation capacity of late passage A-MSCs is quantified in B compared to A-MSCs at passage 5. n=3, *p<0,05, **p<0,01. A-MSCs: adenoid-mesenchymal stem cells, P: passage number, h: hour.

3.5. In vitro aged A-MSCs maintained the migration ability

Wound healing requires the proliferation and migration of many cells including MSCs. Therefore, wound healing as migration ability is an important parameter for MSCs. We compared the wound healing potential of A-MSCs during replicative senescence. For that, we used passage 7 and passage 20 cells and performed the *in vitro* scratch assay on the cells. We observed the migration process under phase-contrast microscopy post 8, 16, and 24 hours of formation of the scratch. The microscopic views were recorded at 0. hours after formation of the scratch and then, the ratio of wound closure at 8, 16, and 24 hours was compared with the result at 0. hours. We observed the significant migration through the decrease in cell-free wound area in passage 7 A-MSCs in the first 8 hours. Besides, the cells at passage 20 did not show high migration potential at 8 hours as well as early passage cells. However, we recorded the close migration potential for the cells at passage 20 to early passage (passage 7) A-MSCs at 24. h. The wound healing ability of A-MSCs during *in vitro* aging was shown as microscopic views (Fig. 5A-H) and quantification graphs (Fig. 5I). These findings show that *in vitro* aged A-MSCs maintained the migration ability after 24h of scratch formation.

3.6. In vitro aging differentially regulated the cycling, apoptosis, and senescence-related genes on A-MSCs

We evaluated the different gene expressions to confirm the effect of *in vitro* aging on cellular processes on A-MSCs. *CCND1* (*Cyclin D1*) and *CDKN1A* (*P21*) genes from the cycling-related genes, the apoptotic gene *BAX* and also, and senescence-related genes *c-Myc* and *p53* were analysed for evaluating of its effect on MSC proliferation, cell death, and senescence during *in vitro* replicative senescence. We found that the expression of *CCND1*, which regulates G1/S transition during the cell cycle, was upregulated 2-fold on A-MSCs at passage 20 compared to A-MSCs at passage 7. Besides, there was not any significant change in the expression of *CDKN1A*, known as a cell cycle inhibitor (Fig. 6). The transcriptional changes on cell cycle genes with *in vitro* aging confirmed the stable proliferation capacity of A-MSCs instead of *in vitro* replicative senescence.

Interestingly, we also found the downregulation of the *p53* gene, which regulates the cell death, nearly 2-fold on A-MSCs at passage 20. The absence of the correlation between *BAX* and *p53* gene expressions shows that the function of the *BAX* apoptotic gene may occur by a p53-independent pathway. Besides, the upregulation *BAX* gene and downregulation of *CDKN1A* and *p53* genes reveal that the A-MSCs at passage 20 may not exhibit the senescence profile in contrast to other sources of MSCs.

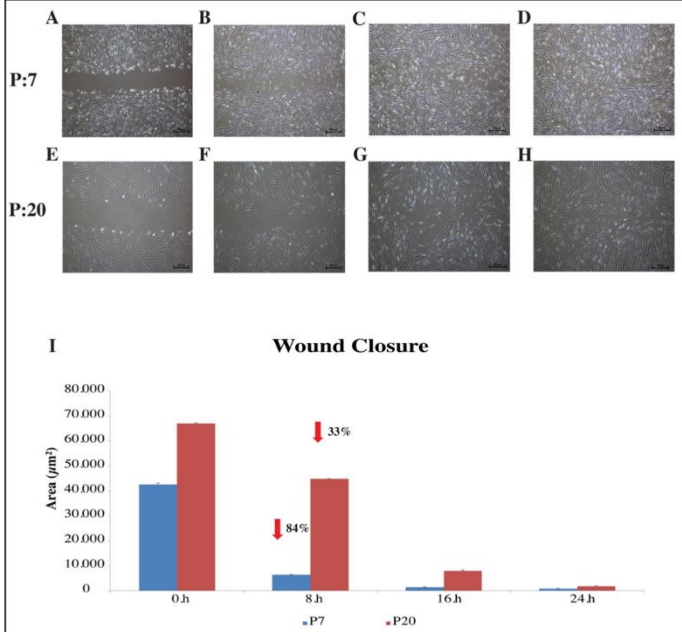

Fig. 5. *In vitro* scratch assay. The bright field microscopy views of A-MSCs post *in vitro* scratch was shown for the observation of A-E) 0.h, B-F) 8.h, C-G) 16.h and D-H) 24.h. I) The quantification of the wound closure is calculated by ImageJ analysis software and also, represented compared to passage 7 cells. Magnification: 100x and scale bar: 100µm H: hour.

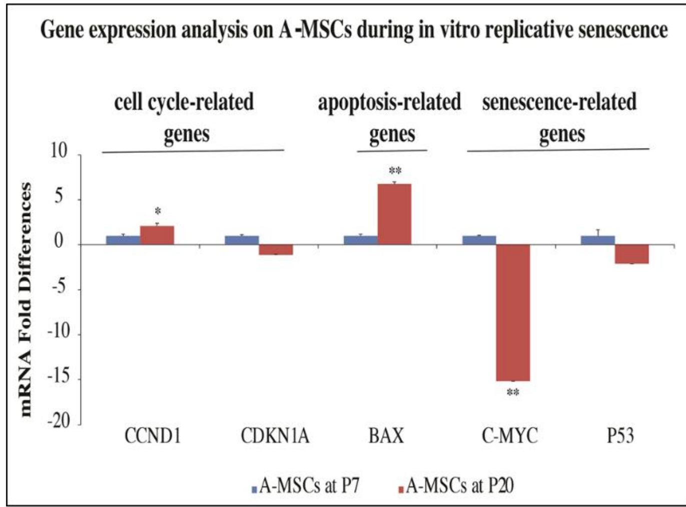

Fig. 6. Gene expression analysis on A-MSCs at early and late passage. The expression level of cell cycle, apoptosis and senescence related genes for A-MSCs at p20 were compared to A-MSCs at p7. The gene expressions were also normalized to *GAPDH* housekeeping gene according to 2-ΔΔCt method. n=2, *p<0,05, **p<0,01.

We found that the expression of *c-Myc* declined 15-fold with the *in vitro* aging process compared to early passage cells. This reduction in *c-Myc* expression was correlated with the decreased growth rate found in our result.

4. Discussion

The immunomodulator and multipotent stem cell properties of mesenchymal stem cells make them important for their therapeutical and clinical use (Gopalarethinam et al., 2023). *In vitro* and *in vivo* studies about the investigation of MSC aging have been carried out until today. The source tissues commonly used for human MSCs are primarily bone marrow and adipose tissue until recent years. The fact that bone marrow is renewable and adipose tissue is easily accessible as waste tissue makes these tissues important. Besides, the different studies showed decreased self-renewal and differentiation potential after *in vivo* and *in vitro* aging of bone marrow and adipose-derived MSCs (Baxter et al., 2004; Stolzing et al., 2008; Zhou et al., 2008; Alt et al., 2012; Wang et al., 2024).

MSCs are a rare cell population compared to the total number of cells in the tissue from which they are isolated. Because of their low numbers, it is important to obtain more MSCs that retain their regenerative ability for their various clinical uses, including regenerative medicine. This is based on the strong proliferative abilities of MSCs *in vitro* (Yang, 2018). Aging occurs with the replicative process and potentially results in reduced stem cell properties and regeneration ability of MSCs. In this study, we investigate the changes in cell morphology, proliferation rate, cell surface antigen expressions, migration and differentiation abilities, and various gene expressions on human A-MSCs that were exposed to senescence *in vitro*. Therefore, MSCs isolated from human adenoid tissue were cultured up to passage 20, and the changes in their morphological features, PDT, the expressions of surface antigens, differentiation potentials, migration abilities, and the cell cycle, apoptosis, and aging-related gene expression profiles were determined due to the increased passages number.

In vitro replicative aged MSCs are reported to have greater morphology and lower spreading potential than young cells (Gu et al., 2016; Gresham et al., 2024). A study reported that after the long-term culture of tonsil-derived MSCs, the cells in passage 15 and passage 2 did not show any significant morphological difference (Choi et al., 2015). In our study, the spindle-shaped cell morphology of A-MSCs with a similar origin to tonsil-derived cells was preserved in the first passages and up to passage 16. As replicative aging progressed, the cells in passage 20 that retained the spindle-shaped morphology were mostly observed, while some had a slightly more irregular morphology. The cell population doubling time in culture varies considerably for *in vitro* senescence. The population doubling time of BM-MSCs has been reported to be significantly prolonged in the later passages, while it is very short in the first passages. In the study, PDT increased to 15.8 days in passage 5 while it was 1.3 days in the first passage (Banfi et al., 2000). According to the results of the study which analyzed the growth characteristics of human umbilical cord-derived MSCs (UC-MSC) after long-term *in vitro* culture, the proliferation rate of cells cultured until passage 17 was significantly reduced to early passage cells. The cells in passage 11, defined as the mid-stage passage, showed a small decrease in their proliferation rate compared to early passage cells (Gu et al., 2016). Similarly, the replicative aging caused a decrease in growth rate in our study. As passage progressed from 7 to 20, the PDT increased from 3.39 days to 5.25 days. The reduction in the growth rate of A-MSCs after *in vitro* replicative aging was similar to T-MSCs (Choi et al., 2015).

The expressions of cell surface antigens are one of the important parameters for the identification of mesenchymal stem cells. It has been reported that (Choi et al., 2015) T-MSCs are highly positive for the expression of CD90, CD44 and CD73 mesenchymal stem cell surface antigens and negative for the hematopoietic stem cell antigens CD34 and CD45. It was not observed that there are no significant differences in the expression of positive and negative surface markers on MSCs after prolonged passages and cryopreservation. According to the results of this study, A-MSCs showed positive expression for MSC-specific cell surface antigens at a high rate similar to T-MSCs until late passages and also showed almost no expression in terms of hematopoietic stem cell antigens. Although there was a statistically significant decrease in CD44 expression, it showed positive expression over 97%. These findings reveal that A-MSCs display a stable profile of MSC marker expressions with *in vitro* aging.

It has been observed that aged BM-MSCs retain significantly the ability to differentiate into adipogenic lineages, while their osteogenic potential is significantly decreased with aging (Yang et al., 2018). The microscopic analysis post Oil Red O and Alizarin Red staining following *in vitro* adipogenic and osteogenic differentiation assays, which were performed to determine the change in differentiation potential of UC-MSCs after *in vitro* aging, showed that adipogenic and osteogenic differentiation capacity was decreased in p11 and p17 compared to p4 cells (Gu et al., 2016). Similarly, in our study, we evaluated the multi-lineage potential of A-MSCs and we found that the lipid vesicles, which are the main indicator for adipogenic differentiation and the osteogenic indicator calcium deposits, were decreased for passage 20 cells compared to passage 4 cells. These findings suggest that the *in vitro* aging suppressed the multi-lineage potential of A-MSCs.

Mesenchymal stem cells are important for their immunomodulatory function through migration to damaged sites. The migration ability varies depending on MSC sources. Recent studies showed that adipose- and bone marrow-derived MSCs had lost their migration ability with cellular aging (Bustos et al., 2014; Liu et al., 2017), while umbilical cord-derived MSCs did not (Naaldijk et al., 2015). Our study observed 85% wound closure after 8 h for early passage cells (passage 7) compared to *in vitro* aged A-MSCs (passage 20). At the end of 24 hours, late passage cells achieved closure of the entire scratch area, similar to early passage, and it was observed that they largely preserved their migration ability at 24 hours.

Finally, we evaluated the different gene expressions to confirm the effect of *in vitro* aging on cellular processes on A-MSCs. The stable proliferation capacity of aged A-MSCs may be explained by the upregulation and slight downregulation of cell cycle regulators *Cyclin-D1* and *CDKN1A*, respectively. In addition, recent studies reported that senescent cells are known to be resistant to apoptosis induced by this genotoxic stress (Rochette and Brash, 2008). In contrast to this information, we found a significant upregulation of the *BAX* gene with *in vitro* aging. Besides, we also found the downregulation of the *p53* gene involved in the regulation of cell death (Amaral et al., 2010) and is the marker for senescence with its function on the regulation of *BAX* and *p21* genes (Zhou et al., 2008) with *in vitro* replicative senescence. The high upregulation *BAX* gene and downregulation of *p21* and *p53* genes reveal that the A-MSCs at passage 20 may not exhibit the senescence profile in contrast to other sources of MSCs. Besides, the absence of the correlation between *BAX* and *p53* gene expressions in our study shows that the function of the *BAX* apoptotic gene may occur by a p53-

independent pathway. In addition, we found that the expression of oncogene *c-Myc* declined 15-fold with *in vitro* aging process compared to early passage cells. It was indicated that overexpression of *c-Myc* induced MSC proliferation and suppressed the multi-lineage differentiation capacity (Melnik et al., 2019). This reduction in *c-Myc* expression was correlated with the slightly decreased growth rate found in our result.

In light of our findings, we indicated that *in vitro* aging is slowly processed on A-MSCs rather than other sources of MSC. Therefore, A-MSCs have more potential for clinic usage depending on their sustainability of MSC characteristics and proliferation and migration abilities with prolonged culture (until passage 20) compared to other sources of MSC. These clinical advantages of A-MSCs make the cells more promising for cellular therapy.

In conclusion, before the clinical use of MSCs, which have an important therapeutic potential in regenerative medicine, it is important to obtain more cells that retain their regenerative ability. It is also known that MSCs obtained from different sources are affected differently by *in vitro* replicative aging. The data from our study showed that A-MSC largely retained the expression of cell surface antigens during long-term *in vitro* culture. However, there is a significant decrease in differentiation potential at passage 20. While no significant difference was observed in the doubling time of cells due to *in vitro* aging, the proliferation ability was significantly preserved. In addition, migration potential was preserved in the 24 hours, although not at the same rate as in the early passage. Therefore, A-MSC will likely be highly promising for future clinical applications with its long-term maintained stemness properties. However, more data and detailed investigations showing the safety and efficiency of A-MSCS are needed for its use in clinics.

Ethical compliance: This study was approved by Ondokuz Mayis University Clinic Research Ethics Committee (Decision number: 2021/352).

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Conflict of interest: The authors declare that they have no conflict of interests.

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Research article

Isolation, identification, and characterization of neopullulanase from *Thermomonas hydrothermalis* GKE 08

Songul Yasar Yildiz*[1]

[1] *Istanbul Medeniyet University, Faculty of Engineering and Natural Sciences, Bioengineering, 34700, Istanbul, Türkiye*

Abstract

The production of neopullulanase from thermophiles, such as *Thermomonas hydrothermalis* GKE 08, has great importance due to the enzyme's unique thermophilic nature. This characteristic results in enhanced stability and functionality at elevated temperatures. It is known that this is a very important issue for industrial processes that require efficient catalysis under extreme conditions. The investigation of pullulanase from *T. hydrothermalis* GKE 08 showed significant results. Optimal conditions for enzyme production were determined, with peak activity observed in the presence of 1.5% soluble pullulan and 0.5% peptone. The study delved into the pH and temperature dynamics, identifying an optimal pH of 7.0 and a temperature of 55°C. Notably, the neopullulanase exhibited time-dependent stability, retaining 72% activity after 1 hour but declining to 50% after 2 hours. Purified pullulanase from *T. hydrothermalis* GKE 08 displayed optimal activity at pH 7.0, with a subsequent time-dependent decline observed during incubation at this pH: retaining 72% activity after 1 hour, approximately 50% after 2 hours, and a significant 77% loss after one day. Furthermore, the enzyme displayed remarkable thermostability at 60°C, with 88% activity after 30 minutes. Metal ion studies indicated susceptibility to inhibition by Cu^{2+}, Mg^{2+}, and Zn^{2+}, while Ca^{2+} stimulated activity up to 138% at higher concentrations. The enzyme's response to specific reagents revealed sensitivity to SDS and EDTA, while urea surprisingly enhanced activity to 85%. The study enhances understanding of pullulanase behavior, offering valuable insights for biotechnological and industrial applications.

Keywords: *Neopullulanase; Thermomonas hydrothermalis; thermophiles; thermozyme*

1. Introduction

Starch, a pivotal biomolecule, serves as a versatile material extensively employed not just in the food processing industry but in many different industries. Traditionally acknowledged for its applications in food, recent technological advancements have expanded its utility in diverse fields, including health, medicine, textiles, paper production, fine chemicals, agriculture and, both petroleum and construction engineering (Egharevba, 2019; Miao and BeMiller, 2023). Within the realm of starch debranching enzymes, pullulanase (EC 3.2.1.41) emerges as a well-known catalyst capable of hydrolyzing not only starch but also other polysaccharides such as pullulan, glycogen, and amylopectin (Boersma, 2024). The vital hydrolytic activity of pullulanase plays a crucial role in applications within the food and pharmaceutical sectors (Hii et al., 2012; Møller et al., 2016; de Souza et al., 2023). Pullulanase (EC.3.2.1.41) plays an essential role as a debranching enzyme, catalyzing the hydrolysis of α-1,6 glucosidic linkages within pullulan, amylopectin, starch, and associated oligosaccharides (Kim et al., 2024). During saccharification processes, this class of enzymes is essential for producing small fermentable sugars by thoroughly breaking down branched polysaccharides (Das and Kayastha 2023).

Pullulanases' unique ability to target α-1,6 linkages is highly beneficial in various settings. In the food industry, it plays

* Corresponding author.
E-mail address: songul.yildiz@medeniyet.edu.tr (S. Yasar Yildiz).
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a role in improving the digestibility and nutritional content of starch-based products like bread and beer (Naik et al., 2023). Furthermore, in pharmaceuticals, pullulanase aids in converting starch into glucose, a step in producing glucose syrups used in medicinal products. Its impact also extends to applications by assisting in the breakdown of starch-based pollutants during bioremediation processes (de Souza, 2010). Additionally, in the textile industry, pullulanase helps efficiently remove starch sizing agents from fabrics during desizing operations, reducing harm (Araujo et al., 2008) The versatility of pullulanase emphasizes its importance across sectors due to its enzymatic functions that support effective processes and sustainable practices. As ongoing research uncovers new uses and improves its properties, pullulanase continues to play a role in advancing industries dependent on starch and its derivatives. The multifaceted contributions of this enzyme underscore its position, in driving technological progress and addressing current industrial challenges.

The pullulanase family exhibits diverse substrate specificity and product profiles, leading to its categorization into five distinct types: (1) Type I, denoted as true pullulanases (EC 3.2.1.41), primarily hydrolyzing α-(1,6) glycosidic bonds and yielding maltotriose; (2) Type II, referred to as amylopullulanases (EC 3.2.1.41), showcasing activity on both α-(1,6) glycosidic bonds in pullulan and α-(1,4) glycosidic bonds in starch, resulting in the production of maltotriose and a mixture of glucose, maltose, and maltotriose, respectively; (3) pullulan hydrolase Type I, recognized as neopullulanase (EC 3.2.1.135), displaying high hydrolytic activity on α-(1,4) and α-(1,6) glycosidic linkages, particularly on pullulan and cyclodextrins, generating panose; (4) pullulan hydrolase II, identified as isopullulanase (EC 3.2.1.57), exhibiting α-(1,4) glycosidic bond hydrolysis on cyclodextrins and yielding isopanose; and finally, (5) pullulan hydrolase III, with a versatile activity spectrum capable of degrading both α-(1,4) and α-(1,6) glycosidic linkages, resulting in a mixture of panose, glucose, maltose, and maltotriose (Kłosowski et al., 2010). Neopullulanase (EC 3.2.1.135), also known as pullulan 4-D-glucanohydrolase, falls within the category of pullulan hydrolase type I and is affiliated with the alpha-amylase family. This enzyme facilitates the breakdown of α-1,4 glycosidic linkages in pullulan, resulting in the formation of panose (6-alpha-D-glucosylmaltose) (Imanaka and Kuriki, 1989; Roy et al., 2003; Bajpai, 2023; Park et al., 2023).

Thermophiles, organisms thriving in elevated temperatures reaching from 41°C to 122°C, predominantly exhibit optimal growth conditions at 80°C (Madigan et al., 1997). These extremophiles are sourced from diverse high-temperature environments, including hydrothermal vents, thermal hot springs, volcanic eruptions, and more, where they produce thermozymes. Thermozymes exhibit enhanced conformational structures, and their adaptability is attributed to the minimal occurrence of additional hydrogen bonds, hydrophobic interactions, and electrostatic interactions (Hussian and Leong, 2023). They have a pivotal role across various industries, including biotechnology, chemistry, food processing, pharmaceuticals, pulp and paper, and the treatment of waste (Bruins et al., 2001; Schäfers et al., 2017; Kumar et al., 2019; Sharma et al., 2019).

Thermozymes have characteristics that are greatly appreciated in industries working with temperatures and difficult environments. In biotechnology, these enzymes are highly valued for their stability and efficiency, in settings that facilitate processes such as DNA amplification (PCR) and protein manipulation. Their resilience is also advantageous in the chemical sector, enabling the catalysis of reactions that would otherwise demand specific conditions or hazardous materials (Giordano, 2010).

Many studies have been conducted to comprehensively study neopullulanases derived from thermophiles for instance *Geobacillus stearothermophilus* TRS40 (Kuriki et al., 1992), *Bacillus stearothermophilus* (Kuriki et al., 1988; Kamasaka et al., 2002; Hondoh et al., 2003; Ece et al., 2015), *Desulfurococcus mucosus* DSM (Jafari et al., 2022), and *Thermotoga maritime* MSB8 (Zhao et al., 2023), as well as hyperthermophiles like *Rhodothermus marinus* (Gomes et al., 2003) and *Thermococcus siculi* HJ21 (Wu et al., 2023). The identification and utilization of pullulanase enzyme have initiated a transformative epoch in various sectors, tackling existing challenges and offering resolutions that contribute to increased efficiency, sustainability, and the production of superior-quality products and processes. This enzyme is poised to outperform in various food processing sectors, heralding a potential reduction in product costs (Naik et al., 2023).

This study is driven by the overarching goal of addressing the crucial role pullulanase plays in biotechnology and the starch industry, particularly in saccharification processes. Great potential of thermophilic pullulanases was investigated for industrial use. Pullulanases, crucial enzymes in the industrial applications, have optimal activity at temperatures exceeding 60°C, making their thermal stability paramount for effective catalysis (Nair et al., 2006).

Given the escalating demand for pullulanase, the primary goal of this study is to identify a novel thermophilic bacterial strain with the ability to produce elevated amounts of this enzyme at high temperatures. The results of this study will contribute to the industry's needs by uncovering a bacterial strain that can meet the growing demand for pullulanase, emphasizing the significance of thermal stability to ensure efficacy in diverse industrial applications.

The genome project of two different strains of *Thermomonas hydrothermalis* is deposited at DDBJ/EMBL/GenBank with the accession number FQUK00000000.1 (isolate: DSM 14834) and JAILZK000000000.1 (isolate: HOT.CON.106). A previous study focused on examining the genomic content of two isolates to identify the presence of glycoside hydrolase enzymes (Yasar Yildiz, 2024). Both isolates were annotated, and the analysis revealed that the neopullulanase (EC 3.2.1.135) enzyme is present in the genome of both strains, serving as the starting point for the present study. Furthermore, within the broader context of this study, the main focus is on isolating neopullulanase from the thermophilic bacterium *T. hydrothermalis*, as there is currently no published information on neopullulanase production from this bacterial strain. The introduction underscores the pivotal role of starch as a widely utilized biomolecule across various industries, underscoring the importance of pullulanase, specifically neopullulanase, in the hydrolysis of starch and related polysaccharides. The growing need, for neopullulanase in starch industries particularly in glucose production, highlights the importance of comprehending its properties and structural features. This study seeks to fill this gap by examining the extraction and analysis of neopullulanase from *T. hydrothermalis* GKE 08 aiming to expand the understanding of neopullulanases found in thermophilic bacteria for use, in various industries.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Bacterial strain *Thermomonas hydrothermalis* GKE 08 was isolated from Golan hot spring, Karakocan, Elazig, Türkiye (Yasar Yildiz, 2024). The 16S rDNA sequence of the bacterial strain was deposited in GenBank with the accession number OQ940533. The strain was incubated on pullulan agar (g/l; pullulan 2; agar 18) and fermented for a duration of 24 hours at a temperature of 55°C. Pullulan degradation was detected through the observation of halo-zones surrounding colonies by covering the agar plate with 99% (v/v) ethanol and letting it stand for 3 hours (Morgan et al., 1979). *T. hydrothermalis* GKE 08 was grown on a basal medium (BM) with the following composition (g/l): peptone 5, yeast extract 2, $MgSO_4$ 0.5, NaCl 0.5, $CaCl_2$ 0.1, glucose 5. Before the medium was sterilized, its pH was adjusted to 7.0. The carbon source was sterilized separately for 3 min at 121°C and mixed with growth media under an aseptic condition (Lee et al., 2001).

2.2. Growth conditions for pullulan production

The pullulan production basal medium (PPBM) contains the same ingredients as BM, except for pullulanase, which is added at a concentration of 5 g/l to replace the presence of glucose. The pH of the PPBM was adjusted to 7.0. *T. hydrothermalis* GKE 08 was cultured in BM for 18 hours under controlled conditions at 55°C, accompanied by agitation at 180 rpm. Subsequently, 1% (v/v) concentration of the BM culture was used to inoculate PPBM. The culture was incubated at 55°C for 48 hours at 180 rpm, following the methods described in previous studies (Nair et al., 2007; Ling et al., 2009).

Growth quantification was achieved through the measurement of optical density at a wavelength of 660 nm in the culture medium utilizing a spectrophotometer (PhotoLab 7100 VIS, Weilheim, Germany) as outlined by Brunswick et al. (1999). This analytical approach facilitated the assessment of microbial proliferation by evaluating the absorbance characteristics indicative of cellular density. Samples obtained periodically were subjected to centrifugation at 5000 rpm for 15 minutes at a temperature of 4°C. The obtained supernatant, devoid of cells, was served as a crude enzyme source for subsequent analyses.

2.3. Enhancing neopullulanase production through culture condition optimization

The impact of variable sugar concentration on production of extracellular enzyme was examined by introducing variable concentrations of soluble pullulan into PPBM. Diverse nitrogen sources, including beef extract, tryptone, peptone, and yeast extract, were individually introduced to assess their effects. The impact of pH and temperature on enzyme production was examined separately, varying the pH and temperature of the production media. Neopullulanase production in the fermentation media was monitored at two-hour intervals until a decline in enzyme activity was observed.

2.4. Pullulanase activity

The procedure established by Kanno and Tomimura (1985) for the determination of pullulanase activity has been modified and conducted at 55°C. The measurement was based on the quantity of enzyme necessary to generate reduced sugars from pullulan, following the protocol of the abovementioned researchers. A 150 µl of supernatant (devoid of cells) was mixed with an equal amount of 50 mM sodium phosphate buffer at pH 7.0, which contained 1% (w/v) pullulan. The same procedures were used to prepare a blank, without the presence of pullulan. The mixtures obtained were incubated at 55°C for one hour, followed by termination of the reaction through the addition of 150 µl of DNS reagent (DNS reagent preparation: 1 g of 3,5-dinitrosalicylic acid dissolved in 20 ml of 2M NaOH, with the gradual addition of 30 g sodium potassium tartrate, and final dilution to 100 ml using distilled water).

After being diluted four times with distilled water, the mixture was boiled for five minutes. Prior to the introduction of the crude enzyme, a blank solution consisting solely of DNS reagent was prepared. Furthermore, a control was established by introducing the DNS reagent before the substrate. The absorbance at 540 nm was used to measure the enzyme activity, following the methods described by Miller (1959) and Kim et al. (2008). The quantity of pullulanase needed to yield-reducing sugar equal to one µmol of glucose per minute under assay conditions was specified as one unit or U/ml (Nair et al., 2007).

2.5. Purification of neopullulanase

The cell-free supernatant obtained from the cultivation broth underwent a process of partial purification using an acetone precipitation method with slight modifications (Obi and Odibo, 1984). To achieve this, 4 volumes of cold acetone were slowly added to 1 volume of the extract, and the resulting mixture was allowed to precipitate overnight at -20°C. After the centrifugation step at 10,000 rpm for a duration of 10 minutes, a resulting pellet was obtained. This pellet was subsequently dissolved in a minimal volume of 0.2 M Tris-HCl buffer with a pH of 8.5.

The subsequent purification steps included anion exchange chromatography, wherein the dissolved sample underwent column chromatography utilizing DEAE cellulose. The fractions eluted from the chromatographic column were meticulously analyzed for enzyme activity. Then all fractions obtained from column chromatography were lyophilized and used for characterization.

2.6. Characterization of pullulan hydrolysis products

Thin-layer chromatography (TLC- TLC Silica gel 60F254, Merck Co, Germany) was used to recognize products that were obtained after the hydrolysis of pullulan. This method provides analysis of hydrolysis products by separating individual components on the chromatographic plates. A 5 µl sample was applied for each spot. A modified version of the method by Raha et al. (1992) was used. An eluent was used which has a mixture of butanol, ethanol, and water in a ratio of 3:1:1 (v/v/v).

The hydrolysis products were observed by spraying the plates with 20% H_2SO_4 and subjecting them to charring for approximately 10 minutes at 100°C. Incubation of the supernatant (devoid of cells) with 1% (w/v) pullulan for 1 hour at 55°C provided reaction products. Along with standard compounds such as glucose, maltose, maltotriose, maltotetraose, maltopentaose, and panose, reaction hydrolysates were applied as spot inoculations onto TLC sheets.

2.7. Biochemical characterization of neopullulanase

The optimal conditions were obtained by investigating influence of varying temperature and pH values on the activity of enzyme. 0.1 mM sodium phosphate buffer was used for the reaction and the reaction mixture was incubated for 1 hour (Duffner et al., 2000; Tang et al., 2008). The pH range of 3.0 to 11.0 was investigated using 0.2 M strength buffers.

In the next stage, time-dependent changes in enzyme activity were observed by investigating the effect of varying pH on enzyme stability. The variation in enzyme stability was observed at temperatures ranging from 0 to 100°C using the standard test protocol at the optimum pH. Additionally, the effect of metal ions on enzyme activity was evaluated. For this purpose, selected metal ions such as Cu^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+} were added to the enzyme at two different concentrations (0.2 mM and 2 mM) and examined with a pre-incubation of 10 minutes. Enzyme activity after this incubation was evaluated separately. Enzyme activity is affected not only by metal ions but also by variable group-specific reagents such as ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and urea. To determine the effect of reagents on enzyme activity, these three different reagents were applied at varying concentrations. The enzyme was individually exposed to each reagent, and subjected to pre-incubation for 10 minutes, and the reaction was initiated by adding the substrate. The resulting activity was then evaluated as previously mentioned. A control was employed using a reaction mixture that lacked metal ions and reagents.

2.8. Statistical analysis

The experimental design employed a fully randomized structure to minimize bias and ensure reliability. Each treatment or sample was evaluated with 3 biological replicates, and the mean ± standard deviation (SD) represents the results that are shown.

The GraphPad Prism was used to analyze the data. The statistical significance of the differences between groups was evaluated using one-way analysis of variance (ANOVA) and independent samples t-tests. Duncan's multiple range test was used at a 95% confidence level for multiple mean comparisons, ensuring that the differences identified were statistically significant with a p-value less than 0.05. This rigorous approach to statistical analysis underlines the robustness and credibility of the study's findings.

3. Results and discussion

3.1. Optimization of culture conditions for maximum neopullulanase production

The highest production of pullulanase was achieved in the existence of soluble pullulan at a concentration of 1.5% (Fig. 1) and with 0.5% peptone (Fig. 2). Previous research on *B. cereus* FDTA-13 has also reported an increase in pullulanase synthesis in the presence of pullulan (Nair et al., 2007). However, substrate repression was reported. When pullulan concentration was elevated to higher levels a decrease in enzyme production was observed. Similar results were also reported for *Bacillus halodurans* (Asha et al., 2013), peak pullulanase activity was observed at a concentration of 1.5%, followed by a decline in enzyme production beyond that concentration. These findings indicate that pullulanase production in microbial systems is not always similar and it shows complex regulation.

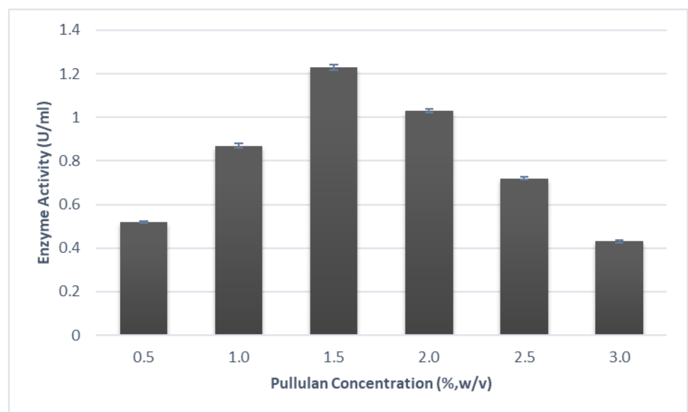

Fig. 1. Effect of different concentrations of soluble pullulan on *T. hydrothermalis* GKE 08 pullulanase production.

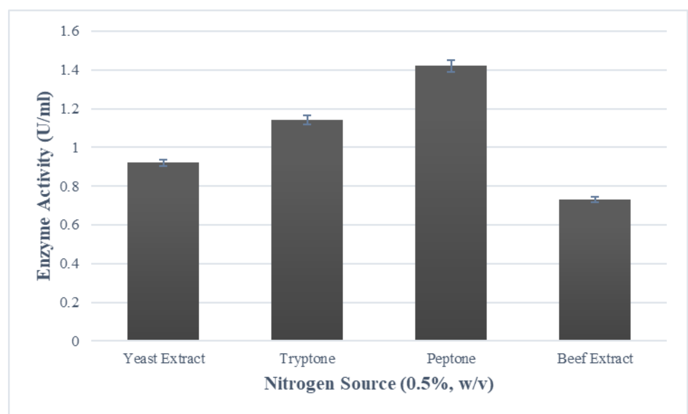

Fig. 2. Effect of different nitrogen sources on *T. hydrothermalis* GKE 08 pullulanase production.

Among the various nitrogen sources examined, peptone proved to be the preferred choice for extracellular pullulanase production. Similar trends were observed in *Clostridium thermosulfurogenes* SV918 (Swamy and Seenayya, 1996) and *B. halodurans* (Asha et al., 2013). The results of these studies are similar to the findings of this study. It was found that the strain, composition of the fermentation medium, and growth conditions have an impact on pullulanase yields. The neopullulanase derived from the *Thermomonas hydrothermalis* GKE 08 strain in this investigation exhibited optimal activity at pH 7.0 (Fig. 3) and 55°C (Fig. 4), which is crucial for the application of the enzyme in saccharification processes. Interestingly, Tang et al. (2008) conducted enzyme screening on soil sediments from a hot spring located in Thailand and reported a novel neopullulanase that is thermally stable. In their study, the neopullulanase displayed the highest activity at 75°C and a pH of 7.0.

Similarly, *Geobacillus stearothermophilus* ADM-11 demonstrated optimal pullulanase activity under identical conditions (Bukhari et al., 2024). *Bacillus amyloliquefaciens* (Castro et al. 1993) showed peak production of pullulanase at a temperature of 44°C and pH level of 5.6. The research conducted by Swamy and Seenayya in 1996 on extracellular pullulanase production by *C. thermosulfurogenes* SV9 revealed the conditions for this process. They identified that the favorable pH range was around pH 7.0 while the best temperature for production was determined to be 60°C. These findings provide insights into enhancing the efficiency of extracellular pullulan-

Table 1
Data of purification process for pullulanase from *T. hydrothermalis* GKE 08.

Fraction	Volume (ml)	Total Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)	Purification Fold	Recovery (%)
Cell free extraction	200	493.64	28.42	17.37	1	100
Acetone precipitation	20	189.43	3.87	48.95	2.61	38.31
Anion exchange chromatography	10	78.21	1.02	76.68	6.31	15.85

ase production by *C. thermosulfurogenes* SV9 making it more suitable for industrial applications. Notably a significant amount of the enzyme totaling 82% was produced at a temperature of 55°C indicating efficiency at this temperature for enzyme production and its potential importance in applications requiring enzymatic activity. Additionally, a study investigated the incubation of *B. cereus* SDK2 under conditions, in the presence of pullulan followed by an assessment of the bacterium's extracellular enzyme activity to understand how varying conditions affect *B. cereus* SDK2 and its enzymatic capabilities when exposed to pullulan (Davaeifar et al., 2015). The unique microbial strain showed healthy growth at a range of pH levels from 5.0 to 9.0 and different temperature conditions, between 30 and 50°C. Its ability to grow vigorously under varying pH and temperature conditions indicates its flexibility and strength. The peak growth performance was particularly impressive at a pH of 7.0 and a temperature of 37°C. This adaptability in thriving under pH and temperature ranges underscores the resilience and versatility of the strain suggesting its potential for use, in environmental and industrial settings.

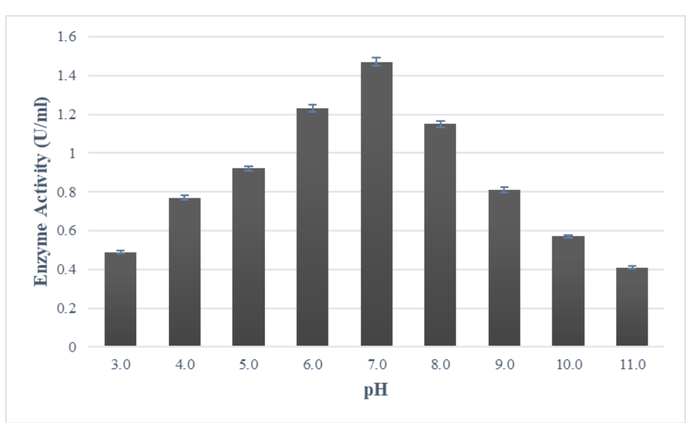

Fig. 3. Effect of different initial pH values on *T. hydrothermalis* GKE 08 pullulanase production.

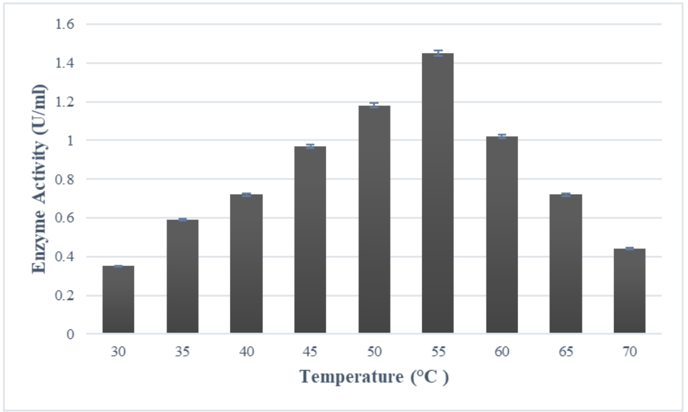

Fig. 4. Effect of temperature on *T. hydrothermalis* GKE 08 pullulanase production.

The isolate was studied under optimal conditions, and different fermentation durations, ranging from 6 to 72 hours, were explored and the 48-hour mark was identified as the time when the highest enzyme production occurs (Fig. 5).

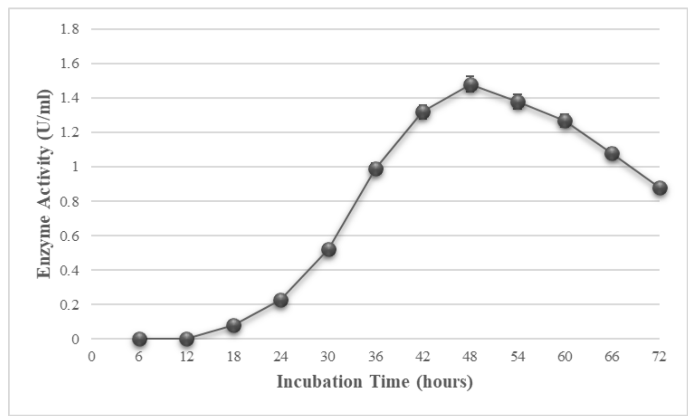

Fig. 5. Time course of *T. hydrothermalis* GKE 08 pullulanase production.

3.2. Purification of neopullulanase

Throughout each stage of the purification process, both the enzymes' specific activity and purification fold consistently showed an increase as outlined in Table 1. This steady progression, during purification highlights how effectively the process enhances the enzymes' activity and overall yield. The systematic improvement in these parameters further confirms the refinement of the enzyme at each purification step demonstrating the reliability and efficiency of the purification method. This affirms that acetone precipitation followed by anion exchange chromatography are techniques for pullulanase enzyme purification. These findings are consistent with research on *Thermus caldophilus* GK-24, where a similar purification process achieved homogeneity in pullulanase resulting in a specific activity of 86.2 U/mg protein and a recovery rate of 13.2% (Kim et al., 1996). The objective of this purification process aimed to extract and enhance pullulanase to a level indicated by the enzyme activity and recovery rate data provided shedding light on the efficiency of the purification method. Similarly, in another study, on *B. halodurans* it was observed to exhibit an activity level of 87.64 U/mg protein with an 8.87% recovery rate (Asha et al., 2013). Another study on pullulanase, from *Bacillus cereus* H1.5 revealed an activity level of 8.987 U/mg, a purification factor of 18.4, and a yield of 10.9% as documented by Ling et al. in 2009.

3.3. Characterization of pullulan hydrolysis products

To verify the identity of neopullulanase, TLC analysis was conducted following an enzymatic reaction with pullulan as the substrate under optimal pH and temperature conditions. For this

purpose, the enzymatic substrate reaction's hydrolyzed products were analyzed through TLC using standard sugar solutions. The TLC analysis revealed that panose was the final byproduct when pullulan was hydrolyzed by *T. hydrothermalis* GKE 08, thereby confirming that the enzyme synthesized extracellularly by *T. hydrothermalis* GKE 08 was indeed a neopullulanase (Fig. 6). The designation of the catalyzing enzyme as a neopullulanase is warranted when panose is the exclusive product recovered from the reaction, as established by previous studies (Labes et al., 2008; Hii et al., 2012; Davaeifar et al., 2015; Balolong et al., 2016).

Panose shows promising potential, as a prebiotic as highlighted in a study by Mäkeläinen et al. (2009). It can also act as a sweetener that helps prevent tooth decay in food products due to its qualities. To begin with, it offers a sweetness making it an appealing substitute for sugars. Moreover, panose resists fermentation by mouth bacteria, which contributes to maintaining health. It hinders the production of glucans from sucrose a process associated with plaque formation. Consequently, panose plays a role in preventing cavities as shown in research by Tsunehiro et al. in 1997. Additionally, a diet rich in carbohydrates, like panose, combined with bacteria can positively impact the human gut system. This duo can improve gut health support a microbiome and potentially enhance digestive function. Findings supported by studies led by Andersen et al. (2012) and Choi et al. (2014).

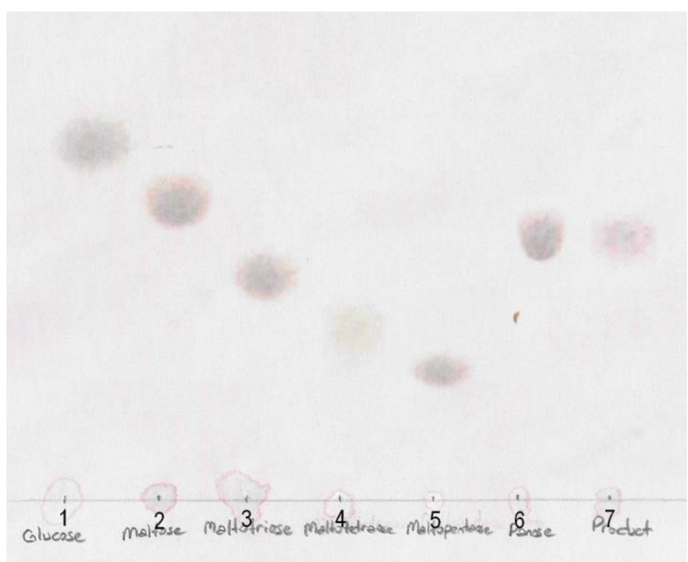

Fig. 6. Pullulan-degrading enzyme profile of *T. hydrothermalis* GKE 08 as determined by TLC: (1) Glucose, (2) Maltose, (3) Maltotriose, (4) Maltotetraose, (5) Maltopentaose, (6), Panose. (7) Product from *Thermomonas hydrothermalis* GKE 08.

3.4. Biochemical characterization of neopullulanase

The investigation into the purified pullulanase, isolated from *T. hydrothermalis* GKE 08, revealed an optimum pH of 7.0, as illustrated in Fig. 7. Further exploration involved subjecting the enzyme to incubation at pH 7.0 for varying durations. After 1 hour, a substantial 72% of the enzyme activity was retained. However, as the incubation period extended to 2 hours, nearly 50% of the enzyme activity was lost. The decline continued with a 77% loss of activity observed after a one-day incubation period, indicating a time-dependent impact on enzyme stability, as represented in Fig. 8.

Examining the temperature influence on the purified pullulanase, the investigation identified a peak activity at 60°C, showcasing the enzyme's thermostable nature, as revealed in Fig. 9. Further characterization of thermostability involved incubating the enzyme at 60°C for an extended duration. After 30 minutes of incubation, an impressive 88% enzyme activity was recorded. The residual activity persisted over time, with 69% observed after the 1st hour, 47% after the 2nd hour, and 21% after the 24th hour, highlighting the enzyme's resilience under prolonged exposure to elevated temperatures (Fig. 10). These findings underscore the importance of understanding the pH and temperature dynamics governing the stability and functionality of the isolated pullulanase, offering valuable insights for potential industrial applications.

Table 2
Analysis of the effect of the differences in metal ions effects on neopullulanase activity of *T. hydrothermalis* GKE 08.

	Relative Activity (%) at two different concentrations	
Reagent	**0.2 mM**	**2 mM**
Cu^{2+}	16	12
Fe^{2+}	75	41
Ca^{2+}	105	138
Mg^{2+}	46	35
Zn^{2+}	51	39
Mn^{2+}	82	57
Control*	100	100

*The control exhibited 100% activity and contained no metal ions. In each case, *p*-values less than 0.05 ($p<0.05$) demonstrated statistical significance.

The activity of the pullulanase enzyme was found to be affected by the presence of metal ions, like Cu^{2+}, Mg^{2+}, and Zn^{2+}. Both Mn^{2+} and Fe^{2+} showed an effect on the enzyme indicating a subtle sensitivity to these specific ions. Interestingly, Ca^{2+} had a stimulating impact on the enzyme activity as shown in Table 2. The pullulanase activity decreased noticeably with concentrations of ions revealing a dose-dependent influence on enzymatic function.

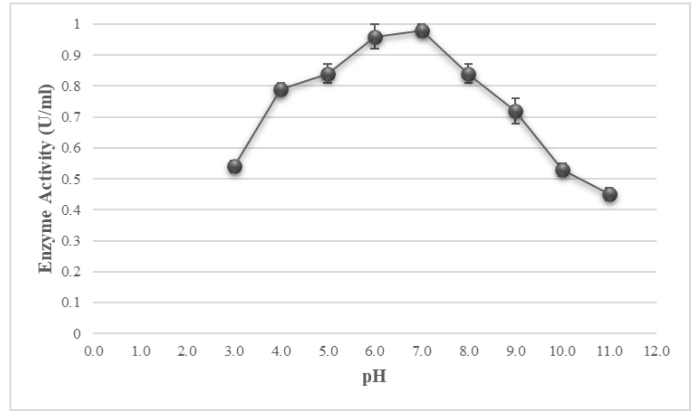

Fig. 7. Effect of pH on *T. hydrothermalis* GKE 08 neopullulanase activity.

Conversely, the introduction of Ca^{2+} ions led to an increase in pullulanase activity highlighting a connection between ion levels and enzyme response. Of interest is the finding that the effect of Ca^{2+} on enzyme activity rose by up to 138% when Ca^{2+} ion concentration was increased tenfold. This indicates an adjustment in pullulanase activity based on the varying levels of Ca^{2+} emphasizing the intricate relationship, between metal ions

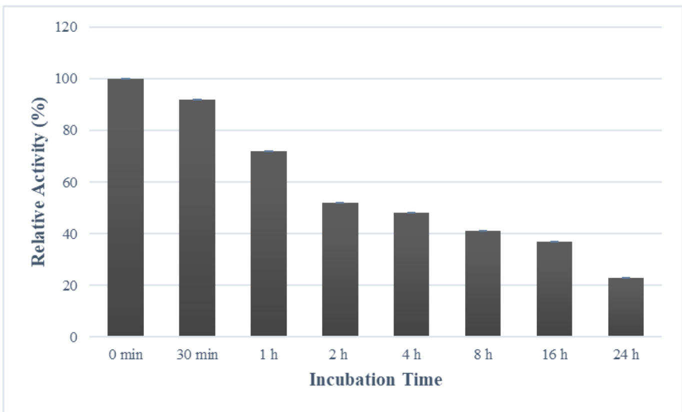

Fig. 8. pH stability of *T. hydrothermalis* GKE 08 neopullulanase at pH 7.0.

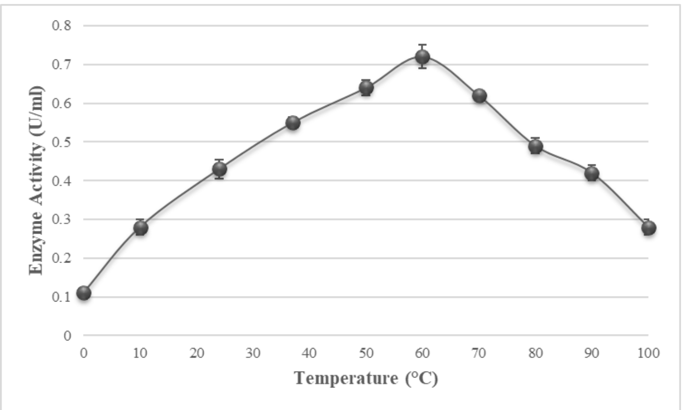

Fig. 9. Effect of temperature on *T. hydrothermalis* GKE 08 neopullulanase activity.

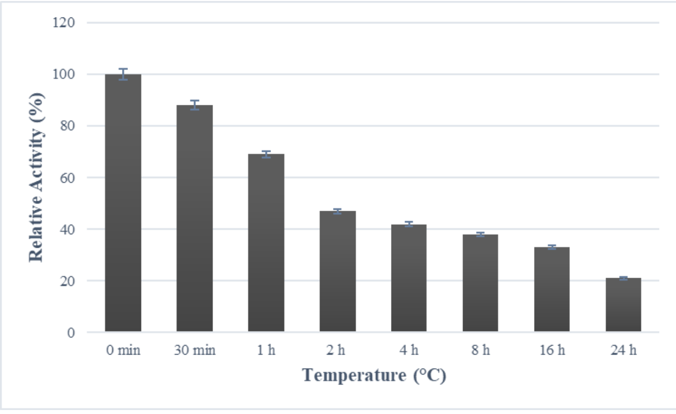

Fig. 10. Temperature stability of *T. hydrothermalis* GKE 08 neopullulanase at 60°C.

and the catalytic efficiency of pullulanase. These findings provide insights into understanding how different metal ions regulate the enzymes' function.

The investigation focused on studying how certain reagents affect the enzymes' performance using pullulan as the material being processed (Table 3). It was observed that when SDS was present, there was a halt in enzyme activity, indicating the inhibitory impact of this substance. On the other hand, when EDTA, a compound known for its ability to bind metal ions was introduced there was a 65% decrease in enzyme activity. This demonstrates the enzymes' susceptibility, to metal ions in the presence of EDTA. However, an interesting discovery was made when urea was introduced. The enzyme exhibited activity levels of around 85%. Urea is commonly used in studies involving changes. Seemed to have a positive impact on enzyme activity in these specific circumstances. The unexpected resilience and enhanced activity raise questions about the stability of the enzyme's structure and its response to substances that modify it. These experiments reveal the detailed nature of interactions between enzymes and specific substances providing insights into their functions. This understanding enhances the comprehension of how enzymes adapt to varying environments opening up applications, in biotechnology and industry.

Table 3
Analysis of the effect of the differences in reagent effects on neopullulanase activity of *T. hydrothermalis* GKE 08.

	Concentration	Relative Activity (%)
EDTA	10 mM	35
SDS	1 mM	0
Urea	0.1 mM	85
Control*	-	100

*The control exhibited 100% activity and contained no reagents. In each case, *p*-values less than 0.05 ($p<0.05$) demonstrated statistical significance.

In a recent study explored the stability and activity of pullulanase derived from *G. stearothermophilus* ADM 11. They focused on how factors like temperature, pH levels, and culture conditions affected the enzymes' performance using pullulan as the substrate. The purified pullulanase exhibited efficiency at 70°C and pH 7.0. Interestingly it remained stable at temperatures up to 90°C showing only a slight 10% decrease in activity at 100°C. The researchers also examined the impact of metal ions on the enzyme activity noting that Ca^{+2} enhanced its function while Sr^{+2} and Ni^{+2} caused deactivation. Mg^{+2} and Mn^{+2} also influenced pullulanase activity to some extent. Additionally, inhibition tests were conducted using substances. The findings revealed that cyclodextrins inhibited the enzyme activity while EDTA and PMSF showed no effect, on its function according to Bukhari et al. (2024).

The study of *B. cereus* strain SDK2 discovered that the presence of calcium ions, Ca^{+2} resulted in an increase, in pullulanase activity within the SDK2 strain (Davaeifar et al., 2015). The inclusion of calcium ions affected how efficiently pullulanase worked in this strain highlighting the role of Ca^{+2} in regulating enzyme performance. This discovery underscores how metal ions interact with enzymes and sheds light on their impact on pullulanase activity in the SDK2 strain. These findings are consistent with a study by Ling et al. (2009) which demonstrated that EDTA hindered pullulanase activity by not forming chelates with divalent cations for enzyme stability and functionality under certain conditions. In their study, they explored how divalent metal ions impact the pullulanase activity produced by *B. cereus* H1.5. The results revealed that, except for Ca^{+2} and Mn^{+2}, the remaining divalent metal ions significantly repressed the activity of the enzyme. Understanding these interactions between EDTA and pullulanase provides insights, for research and practical applications. Similar to this, research findings they noted increased enzyme activity (up to 170%) when Ca^{+2} ions were present leading to improved thermos-activity and thermos-stability. The optimal conditions identified by Ling and colleagues (temperature of 55°C and pH of 6.0) matched with the observations of this study on *T. hydrothermalis* GKE 08 where neopullulanase exhibited peak activity at 60 °C in the existence of Ca^{+2} ions.

The continuous search for effective enzymes in starch

processing, as well as in the food, animal feed, detergent, leather, pulp and paper, textile, medical, and pharmaceutical industries, has prompted extensive investigations into thermophilic or hyper-thermophilic amylolytic microorganisms. The importance of amylolytic enzymes with high thermal stability in the hydrolysis of starch cannot be overstated. The integration of a thermostable neopullulanase, combined with other debranching enzymes and α-glucosidase, presents notable benefits for the starch processing industries. Utilizing collaboration brings benefits improving the efficiency and effectiveness of starch processing. The use of heat, along with neopullulanase enzymes enhances the modification of starch showing promise in transforming practices in the starch processing industry. This advancement is remarkable as it enhances the efficiency, stability, and overall performance of starch hydrolysis processes playing a role in advancing the capabilities and productivity of starch-related sectors. Neopullulanase variants resistant to alkali are utilized in detergent manufacturing.

Furthermore, producing panose with neopullulanase offers an advantage. This is particularly significant as panose is widely recognized as an option in applications. Generating panose with neopullulanase not only increases its versatility but also underscores its impact on gut health and overall, well-being. The association with prebiotic properties positions neopullulanase as a tool for progress in food and nutritional sciences. Diets enriched with bacteria and prebiotic carbohydrates like panose have demonstrated effects on digestive health. Panose, known for its taste possesses qualities that make it an excellent non-cariogenic sweetener for various food applications. Its notable feature lies in its ability to resist fermentation in the mouth leading to health advantages. Panose aids in preventing the formation of sucrose, which helps inhibit bacteria adherence, to tooth surfaces. This versatile feature does not enhance its sweetness. Also highlights its potential as a tooth-friendly sweetener making it an attractive and advantageous option, for various food products. The varied characteristics of panose position it as a choice, for developing tooth sweetener substitutes in the food sector.

4. Conclusion

This study explores the analysis of neopullulanase, from *Thermomonas hydrothermalis* GKE 08 focusing on its production, purification, and enzymatic characteristics. It emphasizes the practicality of this enzyme in applications in processes that involve sugar degradation at elevated temperatures.

The substantial production of pullulanase was successfully accomplished using 1.5% pullulan and 0.5% peptone indicating the regulation of pullulanase production in systems. Among the nitrogen sources investigated peptone was identified as the choice for extracellular pullulanase production. The enzyme from *T. hydrothermalis* GKE 08 exhibited performance at pH 7.0 and a temperature of 55°C aligning with findings from studies on strains like *B. cereus* FDTA 13 (Nair et al., 2007) and *Bacillus halodurans* (Asha et al., 2013). Moreover, this strain displayed growth across a range of pH values (5.0 to 9.0) and temperatures (30 to 50°C) with peak activity observed at pH 7.0 and a temperature of 37°C.

The purification methods employed, such, as acetone precipitation and anion exchange chromatography demonstrated effectiveness in enhancing the purity and activity of neopullulanase. The activity and purification efficiency of the enzyme consistently improved throughout the purification process validating the efficacy of these techniques. The behavior of the enzyme was assessed under conditions including pH levels, time sensitivity and thermal stability. It showed its effectiveness at a temperature of 60°C. It continued to work when subjected to high temperatures for an extended period. The enzyme activity was enhanced in the presence of Ca^{2+} ions whereas ions such, as Cu^{2+}, $Mg^{2+,}$ and Zn^{2+} had varying effects. Additionally, the enzyme reacted differently to substances like urea (which stimulated its activity). SDS/EDTA (which inhibited it) revealing insights, into its characteristics.

Analysis using TLC confirmed that panose was produced as the end product when pullulan was broken down by *T. hydrothermalis* GKE 08 confirming the enzyme as a neopullulanase. Panose's applications as an anticariogenic sweetener highlight the importance of this enzyme in food-related settings.

Future directions may involve genetic engineering strategies to improve neopullulanase production, stability and specificity. Exploring synergies with enzymes or applications in bioremediation processes could open up new avenues for research. This study paves the way for the exploration of neopullulanase offering opportunities for its use, across various biotechnological and industrial fields.

The incorporation of a heat-resistant neopullulanase enzyme, in the processing of starch and various industries could potentially transform the effectiveness and output leading to advancements, in enzyme utilization and industrial biotechnology.

This study sets the stage for the exploration of neopullulanase presenting opportunities for its application across various biotechnological and industrial sectors. Incorporating a heat-resistant neopullulanase enzyme in starch processing and other industries could potentially enhance effectiveness and production output resulting in advancements in enzyme utilization and industrial biotechnology. By leveraging the characteristics of *T. hydrothermalis* GKE 08 neopullulanase industries could achieve sustainable and efficient production processes ultimately driving innovations, in biotechnology and industrial applications.

Conflict of interest: The author declares that she has no conflict of interest.

Informed consent: The author declares that this manuscript did not involve human or animal participants and informed consent was not collected.

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Research article

Novelties in the genus *Trifolium* in Türkiye

Mustafa Keskin*[1]

[1] *Marmara University, Institute of Pure and Applied Sciences, Biology Program, 34722, Goztepe, Istanbul, Türkiye*

Abstract

This article summarizes the information obtained during the revision of *Trifolium* of Türkiye. By the end of the revision, four new taxa (*T. leylae*, *T. konyaensis*, *T. tmoleum*, and *T. hybridum* subsp. *anatolicum* var. *minutum*) were introduced to the scientific world, and *T. dalmaticum* is reported as a new record for the Flora of Türkiye. Three new units of subgeneric classification of *Trifolium* (subsect. *Stipulia*, subsect. *Caudata*, and sect. *Anemopeta*) are offered. Therefore, two new combinations are made (*T. angustifolium* var. *infamia-ponertii* and *T. setiferum* var. *rumelicum*). Distribution information for all analyzed specimens is included in the article. New diagnostic keys for *T. hybridum* species and subgenus *Paramesus* had been constructed.

Keywords: *Keys; new record; new subgeneric units; new taxa; Trifolium*

1. Introduction

With over 255 species, *Trifolium* (Eng.: Clover) is one of the largest genera in the Fabaceae family (Zohary and Heller, 1984; Ellison et al., 2006; Ahmed et al., 2021; Keskin et al., 2023). *Trifolium* is a systematically challenging genus due to its high number of species and genetic diversity; moreover, clovers are one of the most commercially important genera in the family Fabaceae, with at least 16 species used as fodder and green manure (Gillett and Taylor, 2001; Nichols et al., 2023). Clovers are important natural components both in urban environments and in natural grasslands. The species present in this ecosystem play a vital role in maintaining its continuity, productivity, and ecological balance (Ozturk et al., 2012a). In addition, some species of this genus are among the herbaceous species that are often found in a variety of habitats under unfavorable conditions in urban environments (Severoglu et al., 2006; Eskin et al., 2012; Tarakci et al., 2012; Altay et al., 2015).

Trifolium is also an excellent source of pollen for honey production. The presence and diversity of *Trifolium* pollen in honey are a factor that improves the quality of honey. For this reason, it is important to recognize the pollen in honey. Studies for understanding the pollen characteristics of the species of the genus are important in this respect (Keskin 2007a; Ozturk et al 2012b; Kocyigit et al., 2013; Altay et al., 2018). The third volume of Davis' Flora of Turkey contains 94 species, some of which have doubtful or erroneous data (Zohary, 1970). According to the *Trifolium* monograph by Zohary and Heller (1984), the total number in Türkiye is 94, while the world total is 239. Many studies have been carried out on the genus *Trifolium* after the publication of the Flora of Turkey. During these studies, new taxa for the Flora of Türkiye and new species for science were described (Keskin, 2001a; 2001b; 2003; 2004a; 2004b; 2005; 2007a; 2007b; 2011a, 2011b; 2011c; 2012; Keskin et al., 2023). During the preparation of "*Trifolium* of Türkiye", extensive herbarium visits were conducted as part of the revision process. Detailed analyses of all herbaria yielded a wealth of new information. This evaluation led to the discovery of several new species and taxa, the identification of new records for Türkiye, refinements in species descriptions, and the acquisition of new data on some rare species. This study evaluates new data on the genus *Trifolium* and summarizes the results.

2. Materials and methods

During the revision of the *Trifolium* of Türkiye, field

* Corresponding author.
E-mail address: trifolium@hotmail.com (M. Keskin).
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surveys were carried out from 1994 to 2024 and a large amount of data was gathered some of which were new taxa for science and Türkiye.

Despite the use of different floras, articles and collected plants, the new taxa offered in this article could not be recognized (Townsend, 1947; Hossain, 1961; Katznelson, 1965; Katznelson and Morley, 1965; Coombe, 1968; Katznelson and Zohary, 1970; Zohary, 1970; Bobrov, 1971; Meikle, 1977; Zohary and Heller, 1984; El-Macreq, 1986; Oster, 1995; Keskin, 2012; Keskin et al., 2023).

A large number of *Trifolium* samples from various herbaria (E, G, ANK, ANKO, EGE, ESSE, GAZI, HARRAN, ISTE, ISTF, ISTO, MARE, MUFE, NGBB, KNYA, OMUB and VANF) and from the author's clover collection since 1996 were analyzed (acronyms according to Thiers, 2024).

3. Result

3.1. New taxa

3.1.1. Trifolium leylae M.Keskin sp. nov. (Fig. 1, Table 1).

Diagnose: It is related to *Trifolium globosum* L. and *T. batmanicum* Katzn. ex Zohary & Heller. It differs from them in terms of the following characteristics: short length; longitudinally 2-row sparsely patulous hairy, small fruiting heads; fertile flowers 7-12 in a single row; creamish white corolla with violet veined and longer than calyx; seed 2-colored.

Holotypus: Türkiye: Malatya province, Pütürge, Sahilköy area, 27 iv 1999, Mustafa Keskin 2122 (holo. ISTE 78804!).

Paratypus: Türkiye: Malatya province, Pütürge, Sahilköy area, 24 iv 1999, Mustafa Keskin 2087 (ISTE 78769!).

Annuals. Stems 5-10 (-20) cm long and with a few branched; longitudinally 2-row sparsely patulous hairy or rarely subglabrous. Leaves petiolated, 10-40 mm long; leaflets obcordate to cuneate, 10-15 x 0.8-15 mm, pubescence, entire to dentate at the apex. Stipules are ovate to oblique lanceolate, green at apex, paler below, hairy. Heads on a thin or medium sparsely pubescent peduncle at length much longer than subtending petiole, 0.5-25 mm in flowering time, 50-90 mm in fruiting stage. Fertile flowers 7-12, in one (or sub two) lower whorls, deflexed to the peduncle after fertilization, deciduous. The calyx is hairy, 5 mm long, but the tube with 2-colored and sparsely hairy or glabrous in the colored area is nearly twice as long as the teeth. Corolla 8 mm long, creamish white with violet veined on standard, longer than calyx teeth. Sterile flowers as calyx, numerous, apetalous, occurring as a plumose, cylindrical 10-15 mm long body before anthesis, then developing into calyces with solid tube and 5 long deflexed teeth, longer than the tube; the sterile flowers bend over the fertile flowers forming together a discoid body, hairy. The fruiting head 10-20 mm diam. is shed to the soil at maturity, usually in two colors. Fruiting calyx enlarged and teeth slightly elongated. Pods are membranous and enclosed in calyx. Seeds 2-colored, blackish purple and dirty white.

Etymology: The specific epithet of the new species refers to Leyla who is the author's wife and his biggest supporter for sixteen years.

Vernacular name: Since no local name can be observed, the name “ece yoncası” is suggested as a new Turkish Scientific name (Menemen et al. 2021).

Ecology: The habitat of *T. leylae* M.Keskin is a moist area with rich soil. So, it is part of the field. The new species is associated with *Trifolium stellatum* L. var. *stellatum*, *T. cherleri* L., *T. pratense* L. var. *pratense*, *T. campestre* Schreber subsp. *campestre* var. *campestre*, *T. nigrescens* Viv. subsp. petrisavii (Clementi) Holmboe, *T. pauciflorum* d’Urv., *Lathyrus inconspicuus* L., *L. aphaca* L. var. *aphaca*, *L. annuus* L., *Trigonella monspeliaca* L., *Vicia sericocarpa* Fenzl, V. *articulata* Horn., *Cicer pinnatifidum* Jaub. & Spach, *Coronilla scorpioides* W.D.J. Koch, *Medicago orbicularis* (L.) Bartal., *Orchis collina* Banks & Sol., *Cephalanthera damasonium* Druce, *Papaver rhoeas* L., *Sherardia arvensis* L., *Valerianella* sp., *Asperula* sp., *Centaurea* sp..

3.1.2. Trifolium konyaensis M.Keskin sp. nov. (Fig. 2, 3, Table 2).

Diagnose: It is related to *Trifolium ochroleucon* Huds. but differs from it in terms of the following characteristics: different shapes and dimensions of stipules and leaves, small corolla, long peduncles, unique calyx teeth, open calyx throat, etc.

Holotypus: Türkiye: Konya province, Seydişehir-Susuz, Kızpınarı, Suğla lake, wetlands, 1150 m, 16 vii 1980, H. Ocakverdi (Holotype and Isotype KNYA 9897!).

Stems 30-35 cm long and 10-15 mm wide; more branched, woody at base, sparsely appressed or sub-patulous hairy. Leaves in three different shapes. Leaves at base oblong 20-30 x 6-10 mm, retuse at the tip, long adpressed or less patulous hairy, more with many forked veins. Petioles 20-30 mm long, patulous hairy. Stipules 20-25 mm long, tube 10-12 mm, greenish to purple-veined, glabrous; free parts 10-11 mm long, narrowly lanceolate to linear, acute at tips, long patulous hairy, no veins. Leaves at stem alternate except upper leaves, elliptic to lanceolate, obtuse at the tip, 20-30 x 5-8 mm, sparsely long appressed or less patulous hairy. Petioles 20-30 mm long, patulous hairy. Nods long. The upper leaves opposed, 20-35 x 3-5 mm, appressed hairy, narrowly elliptic to lanceolate, acutish at the tip. Petioles are short, at most 10 mm long. Stipules 13-16 mm long. Peduncles 25-75 mm long, appressed hairy. Inflorescences are more flowered, globular to ovoid when young, later ovoid, 18-30 x 15-18 mm. Corolla purple, 8 mm long when young, later 9-10 mm long, obtuse at the tip, longer than calyx. Calyx 6-8 mm, teeth in 3 different sizes; longer tooth 4.2-4.5 mm long, broad at the base, markedly narrowing towards the top, glabrous at the tip with short setae; middle teeth 3.2-3.5 mm; short teeth 2.8-3.0 mm; all teeth green with patulous hairy emerging from a tubercle; densely hairy almost recumbent just below the at base of calyx teeth; the tube straw-colored, 10-thick veined, glabrous at the base, sparsely hairy above, shorter and thinner than the upper hairs. Calyx throat open, ringed with fine hairs. Pods are ovoid, dehiscent with a leathery operculum, glabrous. Young seeds 0.5 mm, ovoid, straw-colored.

Etymology: The specific epithet of the new species refers to Konya province, where the type specimen was collected.

Vernacular name: Since no local name can be observed, the name “Mevlana yoncası” is suggested as a new Turkish Scientific name (Menemen et al., 2021).

Ecology: The nature of the area has been visited and examined in July 2021. The natural habitat of *T. konyaensis* M. Keskin is a moist and fertile ground area. It is a characteristic of the damp meadow.

The new species is associated with *Trifolium pratense* L. var. *pratense*, *T. fragiferum* L. var. *pulchellum*, *T. hybridum* L. subsp. *hybridum* var. *elegans* (Savi) Boiss., *T. hybridum* L.

Table 1
The differences among *T. globosum*, *T. batmanicum,* and *T. leylae*.

	T. globosum **L.**	***T. batmanicum*** **Katz. ex Zohary & Heller**	***T. leylae*** **M.Keskin**
Stem surface	patulous hairy	glabrous	longitudinally 2-row sparsely patulous hairy or subglabrous
Stem length	10-40 cm	20-50 cm	5-10 (-20) cm
Fruiting heads	15-25 mm	20-30 mm	10-20 mm
Fertile flowers	10-16, in two ring	10-14, in two ring	7-12, in single (or sub two) ring
Corolla	white or pale pink, 6-11 mm, slightly longer than calyx	cream color but later becoming rose pink-cream, 5-8 mm, slightly longer than calyx	creamish white and standard violet veined, 8 mm, net longer than calyx
Calyx tube	tube=teeth	tube twice as long as the teeth	tube nearly twice as long as the teeth
Calyx surface	hairy	hairy	hairy, but the tube with 2-colored and sparsely hairy in the colored area
Sterile calyx	only teeth, 8-15 mm	empty calyx and teeth, 10-15 mm	empty calyx and teeth, 10-15 mm
Seeds	yellowish brown	black, ovoid–oblong, rarely white in the hilum area	seed 2-colored, blackish purple, and dirty-white

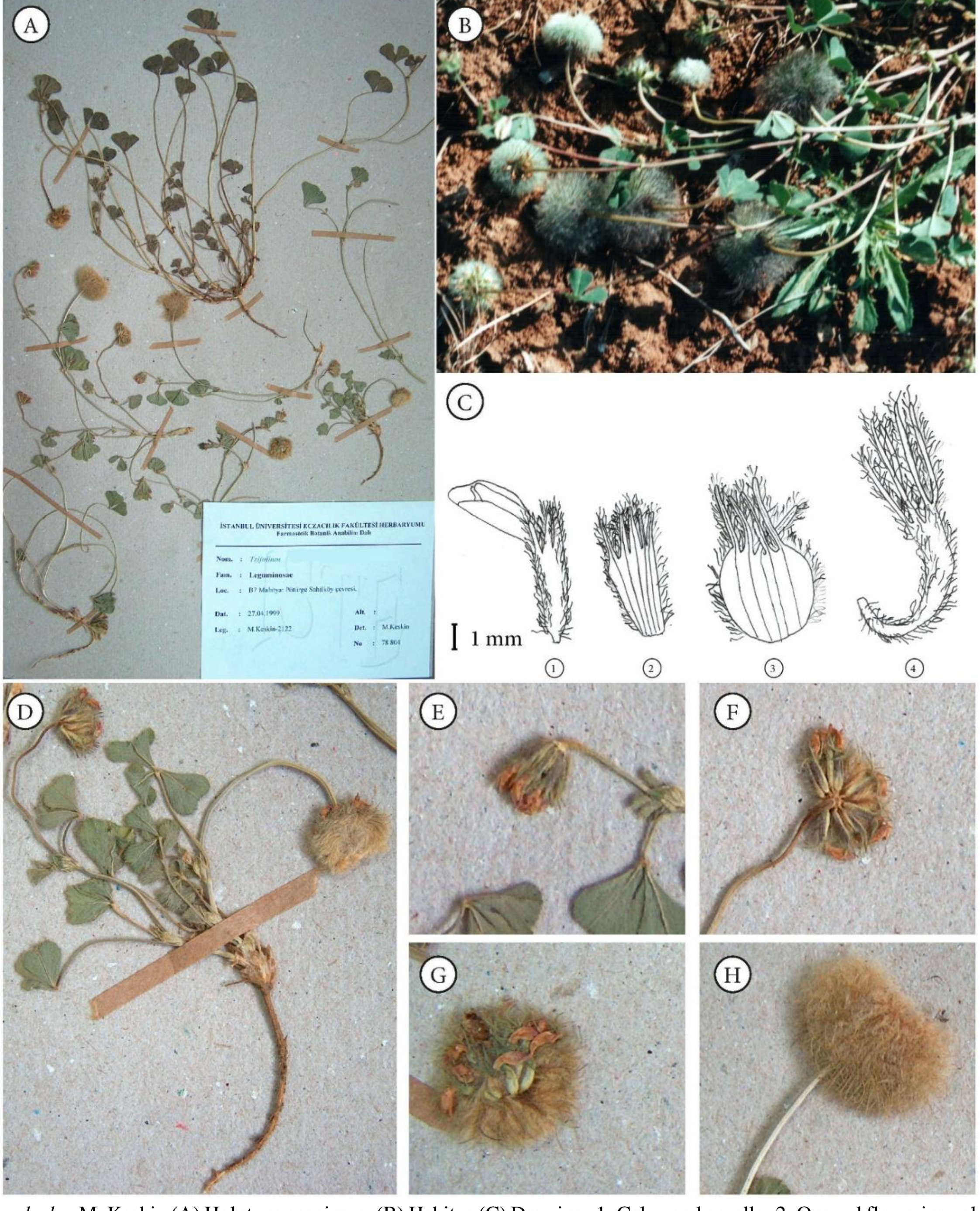

Fig. 1. *Trifolium leylae* M. Keskin (A) Holotype specimen, (B) Habitus (C) Drawing -1: Calyx and corolla -2: Opened flowering calyx -3: Opened fruiting calyx -4: Sterile calyx (D) Single plant (E) Inflorescence (F) Inflorescence with sterile calyx (G) Subinfrutescence (H) Infrutescence.

Table 2
The differences between *T. ochroleucon* and *T. konyaensis*.

	***Trifolium ochroleucon* Huds.**	***Trifolium konyaensis* M. Keskin**
Stem length	20-70 cm	30-35 cm
Stem surface	Appressed or antrorse hairy	Sparsely appressed or sub patulous hairy
Stipules	20-50 mm, free parts subulate	20-25 mm, free parts narrowly lanceolate to linear
Petioles	Lower and middle leaves very long-petioled, upper ones almost sessile	All leaves at least 1.0 cm petioled, absent sessile or almost sessile leaves
Leaves		
at base	Obovate to oblong, cuneate, retuse at apex, 8-16 x 5-10 mm	Oblong, retuse at the apex, 20-30 x 6-10 mm
at middle and upper	Oblong-elliptical to lanceolate, 20-55 x 12-18 mm	Elliptic to lanceolate, obtuse at tip, 20-30 x 5-8 mm and 20-35 x 3-5 mm, sub-appressed hairy, narrowly elliptic to lanceolate
at top	Opposed, narrowly oblong elliptical to lanceolate, shorter than restings, 10-22 x 5-7 mm	Opposed, 20-35 x 3-5 mm, appressed hairy, narrowly elliptic to lanceolate
Heads	Globular to ovoid, later elongating, 10-30 mm across, sessile or on short peduncle subtended by opposite leaves	Globular to ovoid when young, later ovoid, 18-30 x 15-18 mm, 25-75 mm long pedunculate, no subtended by leaves
Flowers	15-18 mm; cream, white, yellowish, pink or purplish	8-10 mm; purple
Standard	Oblong-lanceolate, much longer than keel and wings, acute	Oblong-lanceolate, much longer than keel and wings, obtuse
Calyx	(8-) 10-12 mm	6-8 mm
Teeth of calyx	Lanceolate-subulate with 3-nerved, no setae at the tip	Lanceolate-subulate with 1-nerved and setae present
Throat of calyx	Closed by bilabiate callosity	Open but present a hairy ring
Seeds	Ovoid, brownish	Ovoid, straw color

Fig. 2. *Trifolium konyaensis* M. Keskin: (A) Holotype specimen, (B) Isotype specimen.

subsp. *anatolicum* (Boiss.) Hossain var. *anatolicum*, *Glycyrrhiza echinata* L., *Althea officinalis* L., *Dipsacus laciniatus* L., *Eleocharis quinqueflora* (Hartmann) O.Schwarz, *Galega officinalis* L., *Melilotus albus* Medik., *Lycopus europaeus* L., *Hibiscus trionum* L., *Convolvulus* sp., kindly Poaceae and Cyperaceae species.

3.1.3. A note for Trifolium tmoleum and identification key of subgenus Paramesus

Trifolium tmoleum is accepted as a synonym name in Flora of Turkey (Zohary, 1970) and Genus *Trifolium* (Zohary and Heller, 1984) under *T. glanduliferum* var. *glanduliferum*. However, when the type specimens of both *T. glanduliferum* and *T. tmoleum* taxa were analyzed, significant differences were found to distinguish between the two species. *T. tmoleum* Boiss. is not a valid published name. Firstly, for a species to be valid, its author must also stand behind the name given. Boissier named this species after the name written by Balansa on the herbarium plate: *Trifolium tmoleum* Boiss. but this name has never been validly published anywhere by Balansa. In using this name, Boissier made it clear that he recognized it as a variety (beta) and not as a species: *Trifolium glanduliferum* var. *tmoleum* (Boissier, 1872). If this name had been published as an alternative to the name written by Balansa, it would have been a valid name. In other words, Boissier did not indicate in any way that it was published as an alternative name for "*Trifolium glanduliferum* var. *tmoleum*".

3.1.3.1. Trifolium tmoleum (Boiss.) M. Keskin comb. et stat. nov. (Fig. 4, Table 3).

Syn.: *Trifolium glanduliferum* var. *tmoleum* Boiss., Fl. Orient 2: 141 (1872). *Trifolium tmoleum* Boiss. in Balansa pl. exs.

Lectotypus: Türkiye, İzmir: Yaila de Bozdagh (Tmolus occidental), dans les prairies. 21 Juillet 1854, B. Balansa 175 (as 175. *Trifolium tmoleum*, sp. nov. (Boiss.) (G-Boiss 00783508!). The lectotype is designated here.

Isolectotype: ibid, barcode no "a" !. (Isolectotypus designated here).

ibid: E 00296064 !. (Isolectotypus designated here).

ibid: E 00342454 !. (Isolectotypus designated here).

Examined specimens (Paratypus): Türkiye, İzmir: Yaila de Bozdagh, 21 Juillet 1854, Balansa 414 (G-Boiss 00783511!).

Table 3
The differences between *T. glanduliferum* and *T. tmoleum* according to type specimens.

	Trifolium glanduliferum Boiss.	*Trifolium tmoleum* (Boiss.) M. Keskin
Habit	Spreading and ascending or sometimes prostrate, branched from base	Strict and dichotomously branched at upper, simple at base
Stem length	7-25 cm	30-45 cm
Leaflets	5-8 x 1-2.5 mm	8-15 x 1-2 mm
Peduncles	15-25 mm	12-40 mm
Pedicels	About 1 mm	Absent
Corolla	8-12 mm, two (or three) times as long as calyx	8-10 mm, somewhat longer than calyx or equal
Calyx	4-7 mm	6-8 mm
Teeth at the base	1-nerved	3-nerved

Vernacular name: Since no local name can be observed, the name "Bozdağ üçgülü" is suggested as a new Turkish Scientific name (Menemen et al., 2021).

3.1.3.2. Identification key

1. Involucral bracts absent or very rudiment; leaflets and stipules sparingly or not glandular *T. nervulosum*

1. Involucral bracts present; leaflets and stipules always long glandular

  2. Calyx 3-5 mm; corolla 5-8 mm *T. strictum*

  2. Calyx 4-8 mm; corolla 8-12 mm

    3. Stems branched from the base; calyx 4-7 mm; teeth 1-nerved; corolla 2-3 times longer than calyx *T. glanduliferum*

    3. Stems dichotomously branched at upper part; calyx 6-8 mm; teeth 3-nerved; corolla at most slightly longer than calyx *T. tmoleum*

3.1.4. A revision of Trifolium hybridum

Trifolium hybridum is a widely distributed species with highly variable characteristics. It is an important fodder crop and is widely cultivated for grazing and cutting (Zohary and Heller, 1982). The current classification is no longer adequate for this species. Considering the data obtained to date, the following classification of *T. hybridum* is proposed.

3.1.4.1. Trifolium hybridum L., Sp. Pl. 2: 766 (1753).

Perennials. Stem 1-60 cm, usually almost glabrous or sparsely hairy; erect, creeping, or almost stemless and more branched. Stipules ovate or ovate-lanceolate, lower part adjacent to the petiole, free part long, slender, and narrow. Leaflets elliptic, ovate-elliptic, obovate or more or less rounded, 1-35 x 1-20 mm, the side veins are abundant and prominent; edges whole or finely toothed; the tip is blunt or sometimes slightly recessed. Flowers are usually numerous, usually in globose umbels at the apex, 5-25 mm; peduncles longer than the leaf. Bracts lanceolate, 0.5-1 mm, white, membranous. Pedicel 3-5 mm, fruiting time net curled back. Calyx 5-veined, with 5-faint veins; teeth longer than tube or sometimes equal. Corolla pink or whitish, 7-10 mm; standard elliptic, longer than wings and keels. Ovary with 2 ovules. Pod elliptic. Seed 2, brownish, tuberculate.

3.1.4.2. Identification key

1. Stem long, erect or decumbent, branching few; base not woody …..…..subsp. *hybridum*

  2. Stem hollow with prominent veins; usually more than 25 cm long; inflorescence 2 cm or wider var. *hybridum*

  2. Stem solid, veins absent or less prominent; usually shorter than 25 cm; inflorescence 1-1.5 cm in diameter var. *elegans*

Fig. 3. *Trifolium konyaensis* M. Keskin: (A) Flowering branch, (B) A stipule, (C) The basal leaf, (D) The median leaf, (E) The upper leaf, (F) Calyces and corollas, (G) Close-up of the inflorescence.

Fig. 4. *Trifolium tmoleum* (Boiss.) M. Keskin (A) Lectotype specimen, (B) The close up of inflorescences.

1. Stem short, woody, and abundantly emerging from the base ….…..subsp. *anatolicum*

3. Stems 5–10 cm, prominent on woody stems as well as on flowering branches; leaflets 5–10 mm; leaves overcrowded at the base and not hiding the stem var. *anatolicum*

3. Stems almost absent or sometimes with a few flowering branches prominent; woody stems prominent below the leaves; leaflets 1-3 mm, densely clustered at the base, covering the base like a dome var. *minutum*

3.1.4.3. Trifolium hybridum L. subsp. hybridum var. hybridum

Syn.: *Trifolium fistulosum* Gilib., Fl. Lit. Inch. ii. 86 (1782). *T. bicolor* Moench, Methodus 111 (1794). T. *intermedium* Lapeyr., Hist. Pl. Pyrenées Suppl. 115 (1818). *T. michelianum* Gaudin, Fl. Helv. iv. 573 (1829) non Savi (1798). *T. caespitosum* Eichw., Skizze 166 (1830). Amoria hybrida C. Presl, Symb. Bot. (Presl) i. 47 (1830).

Lectotypus: Habitat in Europae Cultis., Herb. Linn. No. 930.15 (LINN)!. (designated by Zohary & Heller, Genus Trifol.: 145, 1984).

Vernacular name: Melez yonca

Description: The stem is generally hollow; strict, 25-70 cm long; striate; peduncles 2-5 cm, hairy or glabrous; flowering heads 15-20 x 15-20 mm; pedicel hairless sometimes loosely hairy; corolla white or pink, 5-10 mm; calyx 3-4 mm.

Examined samples: Amasya: Akdağ, Ziyaretkar, Saracakağı area, 28 vi 1977, K. Alpınar (ISTE 38034!). **Ankara:** Çubuk, Ovacık-Saraycık village, Hallayık pınarı area, bushes, fountain edge, 1250-1380 m, 11 ix 1992, E. Dündar (GAZI 1484!). **Antalya:** Manavgat, Seydişehir, Irmasan the breach, 1810 m, 04 vii 1976, M. Nydegger 10840 (G!). **Artvin:** Hopa, Sarp, rocky areas across the tunnel, 01 viii 2003, M. Keskin 2994 (ISTE!). **Aydın:** Kuşadası, 5 kilometers north, sea level, 01 vi 1967, A. Huber-Morath 17540 (G!). **Bingöl:** Merkez, 1100 m, 20 viii 1859, T. Kotschy 818 (G 00783573!). **Bolu:** Kale, Kırık plateau, forest clearings, 1550 m, 24 vi 1990, İ. Kılınç (ANKO 1145!). **Eskişehir:** Mahmudiye, vii 1943, H. Demiriz (ISTF 2525!); Oluklu, 03 vii 1963, A. Pamukçuoğlu (EGE 14495!). **İstanbul:** Maltepe, 28 iv 1989, A. Baytop (ISTE 60234!); Sarıyer, Belgrad forest, Büyükbent, 03 vi 1894, G.V. Aznavour (G!); Sultançiftliği, 11 vi 1893, G.V. Aznavour (G!); Şile, Ömerli willage, 06 iv 2004, M. Keskin 3078 (ISTE!); **Kastamonu:** Çatalzeytin, Malafören village, road side, 41° 56′ 205″ K and 034° 13′ 996″ D, 133 m, 17 vi 2008, A.A. Dönmez 14591 (HUB!).

3.1.4.4. Trifolium hybridum L. subsp. hybridum var. elegans (Savi) Boiss., Fl. Orient. 2: 146 (1872).

Syn.: *Trifolium elegans* Savi, Fl. Pis. ii. 161. t. 1. f. 2 (1798). *T. vaillantii* Poir., Encycl. 8: 4 (1808). *T. hybridum* L. var. *pratense* Babenh., Fl. Lusit. 1: 198 (1839). *Amoria elegans* (Savi) C.Presl, Symb. Bot. (Presl) i. 47 (1830).

Lectotypus: [illustration] "*Trifolium elegans*" in Savi, Fl. Pis. 2: t. 1, fig. 2, 1978 (designated by Roma-Marzio & al., *Trifolium* names described by Gaetano Savi, TAXON 67 (2): 413, 2018).

Epitypus: "Monte Pisano sul Monte Aspro (Buti, Pisa), sentiero al margine di un rimboschimento a conifere con sottobosco a prevalenza di eriche, 290 m s.l.m. [WGS84: 43.73702, 10.58162], 29 Jun 2016, F. Roma-Marzio" (PI No. 004813!; Isoepitypus FI!). designated by Roma-Marzio & al., *Trifolium* names described by Gaetano Savi, TAXON 67 (2): 413, (2018).

Vernacular name: Since no local name can be observed, the name "Şirin üçgül" is suggested as a new Turkish Scientific name (Menemen et al., 2021).

Description: Stem is solid, prostrating-ascending, 10-40 cm long; smooth, no vein or obscurely veined but no striate; peduncles 2-10 cm, hairy. Flowering head 15-17 x 15-17 mm; pedicel hairy; corolla pink, red or white-pink, 6-7 mm; calyx 2-3 mm.

Examined samples: Afyonkarahisar: Sandıklı, Akdağ-Sığırkuyruğu around the plateau, 1500 m, 22 vii 1983, M. Nydegger 2390 (EGE 25685!). **Ankara:** Beypazarı, 30 vi 1971, Y. Akman (ANK 884! and G!); Merkez, İstanbul road side, Kargakesmez village, 1120 m, 05 ix 1974, I. Kukkonen 8220 (G!). **Ardahan:** Çıldır on the coast north-west of the lake, near the village of Gölbelen -damp Gladiolus, *Trifolium*, *Polygonum* meadow-, 1960 m, 29 v 1995, A.J. Byfield 1740 (ISTE 69090!). **Artvin:** Hopa, Başköy, mosque relatives, 40° 57′ 16,4″ K and 29° 08′ 43,7″ D, 213 m, 30 vii 2017, M. Keskin 7528 (ISTE!); Hopa, Bucak district, 01 viii 2003, M. Keskin 2952 (ISTE!); Hopa, Bucak district, 1 viii 2003, M. Keskin 2952 (ISTE!); Tiryal dağı, southern slopes, Hatila region, rocky, stream, 1850 m, 28 v 1963, A. Düzenli (ANK 573!). **Aydın:** Çine, Madrandağı, Kavşıt village, 750 m, 2 viii 1978, Ö. Seçmen (1580), G. Görk and all. (EGE 1020!). **Balıkesir:** Kazdağı, Sarıkız tepesi, zirve, 21 vii 2012, M. Keskin 5831 (ISTE!). **Bartın:** Kurucaşile Göçgün seaside, creekside, 24 x 2006, M. Keskin 5297 (ISTE!). **Bilecik:** Pazaryeri, 30 vi 1983, toplayıcısız (ESSE 5947!). **Bolu:** Abant lake around, 02 vii 2009, M. Keskin 5754 (ISTE!); Aladağ plateau, meadow, 1350 m, 11 viii 1985, M. Vural (GAZI 2666!); Karacasu, 700 m, tarihsiz, O. Alpay (ANKO!); Koru motel, mixed *Fagus* and *Abies* forest, 860 m, 30 viii 1972, I. Kukkonen 8237 (G!); Uludağ, experimental pasture, 1300 m, 12 vi 1954, O. Alpay and Gezgürel (G!); Yedigöller national park, fish breeding ground, 900 m, 14 vi 1977, R. İlarslan (HUB 13215! and KNYA 9832!); Yedigölller Milli parkı, Tombullar area, 1500 m, 14 vi 1977, R. İlarslan (KNYA 9930!). Bursa: Uludağ, Arpalı district, 10 km east of Termalin, 140 m, R. Çetik (KNYA 9830!); Uludağ, Çekirge-Uludağ, 31 km, 13 viii 1969, H. Demiriz (ISTF 24558!); Uludağ, Diktekir, 5 vii 1944, M. Başarman (ISTF 4146!); Uludağ, Elmaçukuru, 13 v 1944, M. Başarman (ISTF 2823!); Uludağ, Gökdere, Kestanelik, 28 vi 1944, M. Başarman (ISTF 3492!); Uludağ, Sarıalan plateau, Pala Cemal mangal near, 2 vii 2004, Y. Belge and A. Sarı (ISTE 81879!); Uludağ, Teleferik around, 30 vi 2007, M. Keskin 5706 (ISTE!); Uludağ, Teleferik around, 30 vii 2007, M. Keskin 5706 (ISTE!); Uludağ, summit, 20 viii 1994, S. Alan (ESSE 13314!). Çankırı: Merkez, Research forest, regional 1, 1435 m, 21 vi 1961, İ. Bozakman (ANKO!). **Eskişehir:** Kırka, Türkmen mountain promenade, 28 vi 2007, M. Keskin 5558 (ISTE!); Kırka, Türkmen mountain, Kurugöl, 39° 28′ 08.1″ K and 30° 21′ 24,5″ D, 29 vi 2007, M. Keskin 5637 (ISTE!); Kırka, Türkmen mountain, Kurugöl, 39° 28′ 08.1″ K and 30° 21′ 24,5″ D, 29 vi 2007, M. Keskin 5668 (ISTE!); Kirka, Türkmendağı Kurugöl, 29 vi 2007, M. Keskin 5667 (ISTE!); Kirka, Türkmendağı promenade, 28 vi 2007, M. Keskin 5558 (ISTE!); Sündiken mountain, Güzelce village aroud, Aralık, areas with high ground water, 6 vi 1971, 800 m, T. Ekim (ANK!). **Isparta:** Atabey, Gelincik main hill, alpine

zone, 2200-2700 m, 22 vii 1983, L. Bekat (EGE 35559!). **İstanbul:** Alemdar-Kurtköy, South slopes of Omerli dam, 11 v 1995, A.J. Byfield 2273 and R. Fitzgerald (ISTE 69627!); Akfırat Opposite Formula 1 area, 19 v 2005, M. Keskin 3959 (ISTE!); Alemdağ, 29 v 1898, G.V. Aznavour (G!); Alemdağ, in forest, 17 v 2000, M. Keskin 2404 (ISTE 79232!); Alemdağ, in forest, 25 v 2003, M. Keskin 2857 (ISTE!); Arnavutköy, Durusu, Ormanlı road, 41° 22′ 42,8″ K and 28° 33′ 28,8″ D, 8 m, 19 v 2017, M. Keskin 7148 (ISTE!); Belgrad forest, 29 x 1964, A. Baytop (ISTE 7797!); Beykoz Anadolu Hisarı, 29 v 2005, M. Keskin 4084 (ISTE!); Beykoz, 8 km east, roadside, flowers pink, 29° 10′ D and 41° 10! K, 350 m, 23 vi 1971, J.R. Edmondson (ISTF 25607!); Beykoz, Anadolu hisarı, 29 v 2005, M. Keskin 4084 (ISTE!); Kağıthane-Şişli, 20 vi 1893, G.V. Aznavour (G!); Maltepe, Başıbüyük Mahallesi Süreyyapaşa hospital forest, 04 vi 2006, M. Keskin 5087 (ISTE!); Maltepe, Başıbüyük Mahallesi Süreyyapaşa hospital forest, 01 vii 2000, M. Keskin 2440 (ISTE 79268!); Maltepe, Başıbüyük mahallesi, hill slopes behind the school, 04 vi 2006, M. Keskin 5087 (ISTE!); Paşabahçe-Polenezköy, 02 vi 1940, B.V.D. Post (G!); Pendik, Green fields on the way to Formula One, 41° 02′ 17,9″ K and 29° 18′ 57,5″ D, 206 m, 10 viii 2017, M. Keskin 7583 (ISTE!); Sarıyer, Kale distict, 05 xii 2006, M. Keskin 5229 (ISTE!); Şile, Ömerli village, 6 iv 2004, M. Keskin 3078 (ISTE!); Tarabya, 01 vi 1888, G.V. Aznavour (G!); Tuzla, Akfırat district, Formula 1 area, lake area, 06 v 2005, M. Keskin 3850 (ISTE!); Tuzla, Akfırat beldesi Opposite Formula 1 area, 06 v 2005, M. Keskin 3822 (ISTE!); Tuzla, Akfırat district, Formula door, meadow, 04 vi 2005, M. Keskin 4130 (ISTE!); Tuzla, Akfırat beldesi, Formula One field entrance, meadow, 4 vi 2005, M. Keskin 4130 (ISTE!); Tuzla, Akfırat beldesi, Formula-1, across the runway, 6 v 2005, M. Keskin 3822 (ISTE!); Tuzla, Akfırat beldesi, Formula-1 field entrance, meadow, 19 v 2005, M. Keskin 3959 (ISTE!); Tuzla, Akfırat district, lake area, 6 v 2005, M. Keskin 3850 (ISTE!); Tuzla, Göçbeyli village, 19 v 2005, M. Keskin 3991 (ISTE!); Tuzla, Göçbeyli village, 19 v 2005, M. Keskin 3991(ISTE!); Ümraniye, Alemdağ, 17 v 2000, M. Keskin 2404 (ISTE 79232!); Ümraniye, Alemdağ, 25 v 2003, M. Keskin 2857 (ISTE!). **İzmir:** Bozdağ, 20 vi 1854, B. Balansa 170 (G 00783581!). **Kars:** Ardahan-Kars arası, 30 km before Kars, right of the road, deep valley, inside the waterfall, 1900 m, 20 viii 1984, R.İlarslan (ANK!); Sarıkamış, between Acısu-Sarıkamış, 22 vii 1947, A. Heilbronn, M. Başarman (ISTF 7978!). **Kastamonu:** Hanönü, around Kayabaşı village, 41° 43′ 227″ K and 034° 20′ 566″ D, *Fagus* fores cleanig, A.A. Dönmez 16140 - Z. Uğurlu (HUB!); İnebolu, 2 vii 1947, A. Heillbronn and M. Başarman (ISTF 6986!). **Kırklareli:** Demirköy, 18 vii 1959, A. Baytop and T. Baytop (ISTE 5486a!); Demirköy, 18 vii 1959, A. Baytop and T. Baytop (ISTE 5486a!). **Konya:** Beyşehir, 12 km to Kurucuova, Musalla top, KB slope, 1500-1700 m, 17 vi 1980, M. Serin (KNYA 9831!); Hadim, Bademli village, Kandemir area, çayırlık içleri, 1500 m, 18 viii 1988, M. Serin (KNYA 9833!). **Kütahya:** Gediz, Murat dağı, Karapınar, in forest, on rock, 1650 m, 12 vii 1980, A. Çırpıcı (ISTF 34453!); Simav, 8 km from Simav, from the forest road towards Mount Simav, *Pinus nigra* forest, 1700 m, 17 vii 1965, M.J.E. Coode and B.M.G. Jones 2635 (ISTF 20874!). **Samsun:** Kavak, Azaklı village, 10 vi 2000, M. Keskin 2430 (ISTE 79258!). **Sivas:** Zara, 5 km S of Şerefiye plateau, 1600 m, 19 vi 1958, A. Huber-Morath 15023 (G!).

3.1.4.5. Trifolium hybridum L. subsp. anatolicum (Boiss.) Hossain, Notes Roy. Bot. Gard. Edinburgh 23: 466 (1961) var. anatolicum

Syn.: *Trifolium anatolicum* Boiss., Diagn. Pl. Orient. ser. 1, 2: 31 (1843), non Katznelson (1965). *T. parvulum* Beck ex Stapf, Denkschr. Kaiserl. Akad. Wiss., Wien. Math.-Naturwiss. Kl. 51(2): 379 (1886).

Lectotypus: Türkiye, Tmoli cacumini supra Philadelphiam (Alaşehir), June 1842, Boissier (G-Boiss 00783575!). designated by Hossain, A Revision of *Trifolium* in The Nearer East, Notes Roy. Bot. Gard. Edinburgh 23: 466 (1961).

Vernacular name: Since no local name can be observed, the name "Güdük üçgül" is suggested as a new Turkish Scientific name (Menemen et al., 2021).

Description: Plants branching from the base; stem more or less distinct, creeping or ascending, up to 15 cm; veins on the stem absent or indistinct; peduncles 3-8 cm, hairy or glabrous; flowering heads 10-17 x 10-17 mm; pedicel usually hairy; corolla pink, reddish or purple, 6-10 mm; calyx 3-4 mm.

Examined samples: Afyonkarahisar: Büyükkalecik, Kocatepe, Gölcük district, in water, 1650 m, 14 vi 1982, T. Ekim and H. Malyer (ESSE 2508!). **Amasya:** Akdağ, Alıç plateau, 02 x 1976, K. Alpınar (ISTE 36183!); Akdağ, Ormanözü village, Mezarlık district, Kavacık hill, Zirand road, 01 x 1976, K. Alpınar (ISTE 36164!). **Ankara:** Beypazarı, 30 vi 1971, Y. Akman (ANK 884! and G!); Çamlıdere, Çamkoru research forest, trial area 1435 m, 21 vi 1961, İ.H. Bozakman (ANKO!); Çamlıdere, Çamkoru research forest, trial area 1740 m, 20 vii 1963, İ.H. Bozakman (ANKO!); Çamlıdere, Peçenek, Tatar field, black pine, 1400 m, 2 vi 1978, M. Aydoğdu (ANK!); Çubuk, slopes North East of Karagöl, step, 23 v 1973, S. Erik 23 (HUB 13310!); Karagöl, 64 km south of Ankara, *Pinus nigra* forest,1600 m, 10 vii 1965, M.J.E. Coode and B.M.G. Jones (ISTF 20604!); Kızılcahamam, Çamkoru, cleanig and in forest, roadside, B. Kasaplıgil (ANKO 72!); Kızılcahamam, Soğuksu National Park, Göklü district, 1400 m, 9 vi 1983, A. Güner 4992, K. Sorkun (AEF 12888! and HUB 13308!); Kızılcahamam, Soğuksu National Park, İncegeliş area, cleaning forest, 1550-1600 m, 9 viii 1990, Ö. Eyüpoğlu (GAZI 2040!); between Lalahan-Çiftlik, meadow, 2 vii 1954, R. Çetik (KNYA 16553!). **Antalya:** Gazipaşa, Çobanlar village plateau, Akçamağar district, korunmuş arazi, 1800-2000 m, 20 vii 1981, H. Sümbül 1149 (HUB 13318!); Gazipaşa, Çobanlar village plateau, Dereyurt district, protected area, 1850 m, 12 vii 1983, H. Sümbül 2266 (HUB 13314!); Gazipaşa, Çobanlar village, Oyuklu plateau, 1900-2000 m, 11 vii 1983, H. Sümbül 2230 (HUB 13316!); Gazipaşa, Sugözü village to Ekinçalı plateau, 2000 m, 12 vii 1982, H. Sümbül 1250 (HUB 133171). **Bartın:** Kurucaşile, 24 viii 2006, M. Keskin 5138 (ISTE!). **Bilecik:** Bozöyük, 2 km west of the centre, 21 vi 1990, R. Lampinen 7642 (ISTE 63685!). **Bolu:** Abant lake, 1350 m, 03 viii 1984, Ö. Ödemiş, M. Nydegger, H. Tabaka and Yasuda (EGE 17794!); Aladağ plateau, meadow, 1350 m, 11 viii 1985, M. Vural (GAZI 2622!); Gerede, 5 km west of Yeniçağa, 1040 m, 03 vii 1984, M. Nydegger 19004 (G 386879!); Koru motel, meadow, 850 m, 30 viii 1972, P. Uotilla 20129 (EGE 23466!); Merkez, Aladağ, Değirmenözü experience pasture, 22 vii 1954, O. Alpay (ANKO 193!); Merkez, around hotel, meadow, 10 m, 15 vi 1986, B. Tutel (ISTF 35776!); Mudurnu, Abant lake, 1300 m, 06 vii 1977, M. Nydegger 12013 (G!). **Bursa:** Keşiş mountain, 200 m, 26 v 1899, J. Bornmüller 4352 (G !); Kirazlı, Sarıalan, Elmaçukuru, 28 vi 1944, M. Başarman (ISTF 3573!); Uludağ Teleferik area, 30 vi 2007, M. Keskin 5709 (ISTE!); Uludağ, 09 vii 1978, A.

Baytop (ISTE 40740!); Uludağ, 09 viii 1978, A. Baytop (ISTE 40740!); Uludağ, 7 vii 1963, C. Regel (EGE 1455!); Uludağ, Aras, viii 1945, M. Başarman (ISTF 56331); Uludağ, Bakacık on road, 03 viii 1957, N. Gülen (ISTE 5107!); Uludağ, Bakacıkaltı, 6 ix 1944, M. Başarman (ISTF 4733!); Uludağ, Diktekir, 4 vii 1944, M. Başarman (ISTF 4029!); Uludağ, Elmaçukuru, 28 vi 1944, M. Başarman (ISTF 3524!); Uludağ, Elmaçukuru, around otel, 28 vi 1944, M. Başarman (ISTF 3577!); Uludağ, Karabelen, Dolubaba, 19 v 1944, M. Başarman (ISTF 3074!); Uludağ, Karatepe, Karçukuru, 2 viii 1944, M. Başarman (ISTF 4618!); Uludağ, Kirazlı, Sarıalan, 29 vi 1944, M. Başarman (ISTF 3635!); Uludağ, Kurt rock, viii1945 M. Başarman (ISTF 5687!); Uludağ, Paşaçayırı, 31 vii 1944, M. Başarman (ISTF 4450!); Uludağ, on stream, viii 1945, M. Başarman (ISTF 5654!); Uludağ, around Teleferik, 30 vii 2007, M. Keskin 5709 (ISTE!); Uludağ, peak road, viii 1945, M. Başarman (ISTF 5751!); Uludağ, peak, 13 vi 1953, M. Heilbronn (ISTF 12936!); Uludağ, Zobrandere, 1820 m, 10 viii 1951, H. Demiriz (ISTF 11535!). **Çankırı:** Atkaracalar, Dumanlı mountain, Ulupınar village, erenler district, rocky step, 1200-1400 m, 10 vii 1992, A. Duran (GAZI 1595!). **Düzce:** Akçakoca, between Çiçekpınar-Doğancılar village, along the brooke, 41º 04′ 26″ K and 31º 09′ 39″ D, 20 m, 10 vi 2001, A. Doğru-Koca 1437A (HUB!). **Eskişehir:** Kirka Türkmendağı promenade, 28 vi 2007, M. Keskin 5559 (ISTE!); Merkez Anadolu Üniversitesi Yunus Emre Kampüsü, 29 vi 2007, M. Keskin 5696 (ISTE!). **Isparta:** Senirkent, Garip köy exit, Kapıdağ area, *Cedrus* forest, 1250 m, 9 vi 1983, L. Bekat (EGE 35560!). **İstanbul:** Alemdağ, around Nişantepe, 18 vii 1998, M. Keskin 1915 (ISTE 78609!); Ataşehir, Turkuaz Su Fabrikası-Maltepe Ün. Arası, meadow-maquis, 40º 57′ 28,9″ K and 29º 11′ 32,5″ D, 9 iv 2006, M. Keskin 5947 (ISTE!); Beykoz, Riva area, meadow-maquis, 41º 12′ 15″ K and 29º 15′ 28,5″ D, 17 iv 2016, M. Keskin 6012 ISTE!); Çınarcık, Üçreisler area, 10 viii 1983, E. Tuzlacı (ISTE 52184!); Silivri, Danamandıra Tabiat park road, Sinekli road junction, rejuvenated oak forest, 19 v 2006, M. Keskin 6223, N. Özhatay and E. Özhatay (Sarıyer Orm Müd. !); Silivri, Danamandıra Tabiat park road, Sinekli road, 41º 04′ 24,7″ K and 28º 44′ 27,1″ D, 165 m, 19 v 2016, M. Keskin 6222, N. Özhatay and E. Özhatay (Sarıyer Orm Müd.!); Şile, İmam Hatip Lisesi near, meadow-maquis, 41º 02′ 55,7″ K and 29º 18′ 03,1″ D, 130 m, 23 iv 2016, M. Keskin 6057, N. Özhatay and E. Özhatay (Sarıyer Orm. Müd.!). **Kahramanmaraş:** Engizek mountain, Kavurmaçukuru area, high mountain meadows, 2200-2300 m, 20 vii 1987, H. Duman (GAZI 3636!). **Kastamonu:** Taşköprü, Kayabaşı village area, climb the fire tower, 41º 33′ 110″ K, 034º 12′ 183″ D, mountain steppe, *Pinus nigra-Pinus sylvestris* açıklığı, 1710 m, 04 viii 2010, A.A. Dönmez 17571, Z. Uğurlu (HUB!). **Kayseri:** Akkışla, Ganişeyh, Hınzır mountain, Göğkuyu area, 1900 m, N. Çelik (HUB 13311!). **Konya:** Akşehir, Sultandağları, Tekke around, North of the coal hills, 2080 m, 28 vii 1976, G. Dökmeci (ISTE 35556!); Bozkır, Ardıç spring, 1800 m, 15 vi 1967, R. Çetik, T. Ekim and E. Yurdakul (KNYA 9829!); Ereğli, Aydos mountain, Berendi, Meydan plateau, alpinic step, 2500 m, 23 vi 1977, S. Erik 2302 (G!); Ereğli, Aydos mountain, Berendi, Meydan plateau, alpinik step, limestone bedrock, 2500 m, 23 vi 1977, S. Erik (HUB 13299!); Tavşan hill, *Quercus* forest, 1350 m, 26 v 1989, A. Tatlı, B. Eyce and M. Serin (KNYA 9828!). **Kütahya:** Tahtalı mountain, 1600 m, 9 vi 1981, G. Görk and M. Nydegger (EGE 38026!). **Malatya:** İnönü Üniversitesi campus, 15 vii 2006, M. Keskin 4992 (ISTE!). **Mersin:** Aslanköy, Dümbelek pass, 37º 06′ 24,6 K and 34º 15′ 47,2 ″ D, 1891 m, 1 viii 2008, M. Keskin 4565 (ISTE 83286!). **Muğla:** Sandras mountain, Çiçekbaba 2100 m, 14 vii 1978, N. Özhatay and E. Özhatay (MUFE 4229!). **Niğde:** Bulgar mountain, 2100-2500 m, 04 vi 1853, T. Kotschy 361a (G 00783580!). **Rize:** İkizdere, below the Haya village, Spruce-Fir clearing, 1650 m, 6 viii 1986, A. Güner 7084 (HUB 13214!). **Sivas:** Şarkışla, Diğnendim hill, 39º 32 ′ 49″ K and 36º 08′ 21″ D, alpinic meadow, 1900-2000 m, 25 x 2007, B. Özüdoğru 1572 (HUB!). **Tokat:** Artova, Çamlıbel, Yıldızeli, 1700 m, 14 vi 1939, H. Reese (G!); Reşadiye, Karlı plateau, 27 vii 1972, Ş. Şahin (ISTE 23343!). **Trabzon:** Meryemana area, Gırlav plateau, slope, 17 viii 1980, A. Okatan (ISTE 48344!). **Yozgat:** Milli park, under *Pinus nigra*, 1500 m, 26 vi 1979, B. Saygın (ANK 54!).

3.1.4.6. Trifolium hybridum L. subsp. anatolicum (Boiss). Hossain var. minutum M.Keskin var. nov. (Fig. 5)

Holotypus: Türkiye, Muğla: Sandras Dağ, W side of the summit area, 2100-2200 m, Snowbed meadows and rocky slopes, Serpentine. Prostrate in snowbed meadow. Flowers pink. 37º 04′ N and 28º 50′ E, P. Hartvig, Ö. Seçmen, A. Strid 23352 (holo. EGE 27963!).

Vernacular name: Since no local name can be observed, the name “doruk üçgül” is suggested as a new Turkish Scientific name (Menemen et al., 2021).

Diagnose: It is closed to *T. hybridum* L. subsp. *anatolicum* (Boiss.) Hossain var. *anatolicum* but stems obscure and very rich branched; the flowering branch is short and a few. The leaves come out in dense bunches and cover the body like a dome. Leaflets 1-3 mm, usually orbicular. Peduncles 1-5 cm, hairy.

Description: The stems are indistinct, thick woody, 1-4 cm, but not visible due to dense leaves. Flowering branch 1-5 (-10) cm. Leaves come out in bundles. Leaflets almost orbiculate or slightly obovate–obcordate, 1-3 mm diam.; minutely serrate at the margin, veins little obvious and a few. Petiols are loose hairy. Stipules mebranous at base, up to 1 cm; upper ones green, 0.5 mm. Pedicel hairy, 5 mm. Calyx 5-veined, almost membranous, other 5-veins obscured; tube hairy at base. Calyx teeth are as long as tube, obtuse at the tip. Calyx 3 mm. Bracts membranous 1 mm. Corolla 5-6 mm, pink-light purple. Seeds 2, 1.5 mm, almost orbiculate and depressed not spheroidal. Legumes are thick veins and slightly longer than tubes, shortly hairy. Peduncles 1-5 cm, hairy.

Examined samples: Antalya: Akdağ, 1700-2000 m, 8 vii 1993, M. Nydegger, N. Özel, G. Görk (EGE 26643!); **Isparta:** Atabey, Gelincik main hill, alpine zone, 2200-2700 m, 22 vii 1983, L. Bekat (EGE 35559)!. **Muğla:** Sandras mountain, west slope, serpentine land, 2100-2200 m, 07 vii 1984, P. Hartwig, Ö. Seçmen & A. Strid 23352 (G 284827!); Sandras mountain, Dikmecik plateau, 1710 m, 3 viii 1978, Ö. Seçmen (EGE 17110!); Sandras mountain, south west, *Pinus nigra* forest, 37º 04′ K, 28º 49′ D, 1750 m, 6 vii 1984, P. Hartvig, Ö. Seçmen, A. Strid 23324 (EGE 27959!).

3.2. Two New Combinations

3.2.1. Trifolium infamia-ponertii Greuter

Trifolium angustifolium L. is a common species worldwide. It exhibits various different morphological characters. Therefore, different conceptions have been put forward from time to time to determine the limits of this species.

Fig. 5. Holotypus of *Trifolium hybridum* L. subsp. *anatolicum* (Boiss). Hossain var. *minutum* M.Keskin (EGE 27963).

For this historical process, it is sometimes encountered with name confusion during the naming of this species. *T. angustifolium* L. var. *intermedium* Gib. & Belli and *T. intermedium* Guss. have been described as a variety of this species. Later, a researcher discovered this nomenclatural confusion and proposed a new name for this plant: *Trifolium infamia-ponertii* Greuter (Greuter, 1976).

Although Greuter accepted it as a separate species, it has become clear over the years that this plant does not normally have sufficient morphological differences to be clearly distinguished from *Trifolium angustifolium* L. For this reason, it was concluded that it would be better to consider this name at the varietal level of *Trifolium angustifolium* L..

***Trifolium angustifolium* L. var. *infamia-ponertii (*Greuter) M. Keskin, comb. et stat. nov.**

Syn.: *Trifolium intermedium* Guss., Cat. Pl. Hort. Reg. Bocc.: 82 (1821), nom. illeg. non Lapeyr (1818). *Trifolium angustifolium* L. var. *intermedium* Gibelli & Belli, Mem. Reale Accad. Sci. Torino, ser. 2, 39: 420 (1889). *Trifolium angustifolium* L. subsp. *intermedium* (Gibelli & Belli) Arcang., Comp. Fl. Ital., ed. 2: 495 (1894). *Trifolium angustifolium* L. var. *acrogymnum* Maire, Pl. Marocc. Nov. 1: 3 (1929). *Trifolium angustifolium* L. var. *acrolophum* Maire, Pl. Marocc. Nov. 1: 3 (1929). *Trifolium angustifolium* L. subsp. *gibellianum* Pignatti, Giorn. Bot. Ital. 107: 217 (1973). *Trifolium angustifolium* L. subsp. *intermedium* (Guss.) Ponert, Feddes Repert. 83, 9/10: 637 (1973). nom. illeg. *Trifolium infamia-ponertii* Greuter, Candollea 31: 215 (1976).

3.2.2. Trifolium rumelicum Griseb.

Trifolium rumelicum was analysed as a variety of *T. vesiculosum* in the Flora of Turkey. (Zohary, 1970; Ovalle et al, 2010). However, as a result of the studies on the types of this species, it was concluded that it is close to *T. setiferum*. For this reason, it was concluded that *T. rumelicum* species should be related to *T. setiferum* species.

***Trifolium setiferum* Boiss. var. *rumelicum* (Griseb.)M. Keskin comb. et stat. nov.**

Syn.: *Trifolium vesiculosum* var. *rumelicum* Griseb.; Spic. Fl. Rumel. 1: 35 (1843). *Trifolium rumelicum* (Griseb.) Halácsy; Consp. Fl. Graec. 1: 399 (1900).

3.3. Some notes about the infrageneric classification of Trifolium subgen. Trifolium sect. Trifolium

3.3.1. Correction of subsection Stipulia

Subsection *Stipulia* was previously published by Keskin et al. (2023) but is not valid because the type information was not written. It is redefined here by giving the correct type of information.

Subsect. *Stipulia* M. Keskin, subsect. nov.

Diagnose: It differs from the other subsections of sect. *Trifolium* in terms of the following characters: free part of stipules ovate-lanceolate to lanceolate, green, foliaceous; longest tooth of calyx 3-5 nerved.

Vernacular name: The name "Taraküçgülü altseksiyonu" is suggested as a new Turkish Scientific name (Menemen et al., 2021).

Typus: *Trifolium longidentatum* Nábělek

The members of subsection *Stipulia* M. Keskin are as follows:

1) *Trifolium longidentatum* Nábělek, Spisy Přír. Fak. Masarykovy Univ. 35: 69 (1923).
2) *Trifolium kurdistanicum* S.Yousefi, Assadi & Ghaderi, Phytotaxa 297(2): 217 (2017).
3) *Trifolium elazizense* M.Keskin, Sonay & Balos, Phytotaxa 583(2): 202 (2023).

3.3.2. A new subsection of Trifolium

Two *Trifolium* species endemic to Türkiye should be combined under a new sub-section due to their common characteristics.

Subsect. *Caudata* M.Keskin, subsect. nov.

Diagnose: Perennials. The leaf crowded at the base. The calyx is 10-nerved; the mouth closed by two-lipped callosity; teeth are 1-nerved, different lengths. Corolla longer than calyx.

Vernacular name: The name "Anaüçgül altseksiyonu" is suggested as a new Turkish Scientific name (Menemen et al., 2021).

Typus: *Trifolium caudatum* Boiss.

The members of subsection *Caudata* M. Keskin are as follows:

1) *Trifolium caudatum* Boiss., Diagn. Pl. Orient. ser. 1, 9: 22 (1849).
2) *Trifolium davisii* Hossain, Notes Roy. Bot. Gard. Edinburgh 23: 403 (1961).

3.3.3. A new section and a short note for subgenus Calycomorphum

The subgenus *Calycomorphum* shows distinct characteristics within the genus *Trifolium* and even within the family. Some of its flowers are sterile, containing no petals, stamens or ovaries, only empty calyx tubes or long teeth. The seed heads either penetrate deep into the soil or are in the form of a hairy ball.

Subgenus *Calycomorphum* (C. Presl) Hossain, Not. Roy. Bot. Gard. Edinb. 23 : 438 (1961).

Syn.: *Calycomorphum* C. Presl, Symb. Bot. (Pragae) 1: 50 (1831). Sect. *Trichocephalum* Koch, Syn. Fl. Germ. Helv. 171 (1835). Sect. *Calycomorphe* (C. Presl) Griseb., Spicil. F. Rumel. 1 : 31 (1843). Sect. *Oliganthema* Bertol., Fl. Ital. 8 : 131 (1850), pro parte.

Diagnose: Annuals. Flowers sessile and without stalks; inner flowers in sterile, petalless calyx or long teeth; outer flowers fertile; legume 1-seeded.

Vernacular name: The name "Yeraltıüçgülü altcinsi" is suggested as a new Turkish Scientific name (Menemen et al., 2021).

Typus: *Trifolium subterraneum* L.

Sect. *Calycomorphum*

Syn.: Strips *Subterranea* (Carpohypogea) Gib. et Belli in Mem. Accad. Sc. Tor. II, 43: 181 (1893). Strips (*Medusea*) Gib. et Belli subsect. *Geotropa* Gib. et Belli in Mem. Accad. Sc. Tor. II, 43: 205 (1893).

Diagnose: Short, decumbent plants; sterile flowers appear after flowering; fruiting heads touch the soil or penetrate completely into it and develop there, never forming a hairy ball.

Vernacular name: The name "Yeraltıüçgülü seksiyonu" is suggested as a new Turkish Scientific name (Menemen et al., 2021).

Typus: *Trifolium subterraneum* L.

The members of section *Calycomorphum* are as follows:

1) *Trifolium suberraneum* L., Sp. Pl. 2: 767 (1753).
2) *Trifolium israeliticum* D. Zohary & Katznelson, Austral. J. Bot. 6: 179 (1958).

Sect. *Anemopeta* (Gib. et Belli) M. Keskin stat. nov.

Syn.: Sect. *Calycomorphum* gruppo *Carpoepigea* (Medusea) subsect. *Anemopeta* Gib. et Belli in Mem. Accad. Sc. Tor. II, 43: 207 (1893). Sect. *Calycomorphum* subsect. *Anemopeta* Katznelson & Morley in Israel J. Botany 14, 112, 171 (1965).

Diagnose: Plants usually grow more or less erect, fertile and sterile flowers develop almost simultaneously; the fruit is like a hairy ball and never penetrates below the ground.

Vernacular name: The name "Yumakyonca seksiyonu" is suggested as a new Turkish Scientific name (Menemen et al., 2021).

The members of section *Anemopeta* (Gib. et Belli) M. Keskin are as follows:

1) *Trifolium batmanicum* Katznelson ex Zohary & Heller, Genus Trifolium 520 (1984).
2) *Trifolium chlorotrichum* Boiss. & Balansa, Diagn. Pl. Orient. ser. 2, 6: 48 (1859).
3) *Trifolium eriosphaerum* Boiss., Diagn. Pl. Orient. ser. 1, 9: 25 (1849).
4) *Trifolium globosum* L., Sp. Pl. 2: 767 (1753).
5) *Trifolium leylae* M. Keskin, sp. nov., in this article.
6) *Trifolium meduseum* C.I. Blanche ex Boiss., Fl. Orient. 2: 134 (1872).
7) *Trifolium pauciflorum* d'Urv., Mém. Soc. Linn. Paris 1: 350 (1822).
8) *Trifolium pilulare* Boiss., Diagn. Pl. Orient. ser. 1, 2: 29 (1843).

3.4. A new record for Türkiye

***Trifolium dalmaticum* Vis., Flora 12 (1, Ergänzungsbl.): 21 (1829).**

Annuals. Stems 10-25 cm, patulous in the lower part and adpressed hairy in the upper part; more or less striate. Lower leaves are long-petioled, and those at the middle and apex of the stem are sessile or sometimes subsessile. Stipules oblong-linear, membranous with obviously nerved; free parts long patulous hairy. Leaflets 10-15 x 6-10 mm, cuneate, orbicular, denticulate, nerves recurved near margin; those of lower leaves obovate or spherical, those of upper ones obovate-oblong. Flowering heads terminal, 10-30 x 8-10 mm, involucrate by upper leaves and its stipules. Flowers many 10-12 mm long. Calyx tube 10-nerved, generally hairy; teeth sparsely hirsute, lanceolate-subulate to narrow-linear, rigid, unequal; fruiting calyx somewhat large, teeth indurating, stellately spreading; throat narrowed by a callous ring. Corolla pink is 1.5-2 times longer than calyx. Legume ellipsoid and somewhat compressed, membranous. Seed 1-1.5 mm, obovoid.

Examined specimens: İstanbul: Erenköy, 07 v 1891, *G.V. Aznavour* (G!) and Kağıthane, 19 vi 1978, *G.V. Aznavour* (G!).

Vernacular name: Since no local name can be observed, the name "Kaba yonca" is suggested as a new Turkish Scientific name (Menemen et al., 2021).

4. Discussion

Türkiye is a rich country in terms of *Trifolium* species and is home to almost half of the world's species. Especially recent studies have shown that this genus is definitely monophyletic (Ellison et al. 2006, Watson et al. 2000).

In this paper, three new taxa are described for science in the light of specimens collected from Türkiye: *T. leylae* M. Keskin (Subgenus *Calycomorphum*), *T. konyaensis* M. Keskin (Subgenus *Trifolium*), and *T. hybridum* L. subsp. *anatolicum* (Boiss.) Hossain var. *minutum* M. Keskin (Subgenus *Lotoidea*). The taxonomic status of the species *T. tmoleum* was also clarified. Since it was realized that it could not be a synonym of *T. glanduliferum* and that it has unique characteristics, this species was reinstated: *T. tmoleum* (Boiss.) M. Keskin (Subgenus *Paramesus*). The subgeneric classification of the genus *Trifolium* was investigated. As a result, three new classification units were proposed to science: subsect. *Stipulia* M. Keskin, subsect. *Caudata* M. Keskin, and sect. *Anemopeta* (Gib. et Belli) M. Keskin. In addition, two new taxonomic arrangements were made: *T. angustifolium* var. *infamia-ponertii* (Greuter) M. Keskin and *T. setiferum* Boiss. var. *rumelicum* (Griseb.) M. Keskin. Finally, the presence of a new *Trifolium* species not previously reported in Türkiye is reported: *T. dalmaticum* Vis.

Keskin (2012) previously reported 106 *Trifolium* species in the flora of Türkiye. Since it was concluded that *T. bithynicum* Boiss. is actually a synonym of *T. medium* L., and since there was no evidence for the presence of *T. isthmocarpum* Brot., *T. mutabile* Port., *T. plebium* Boiss., and *T. rubens* L., whose presence in Türkiye was already doubtful, they were removed from the list of Turkish flora.

It is concluded that the number of species belonging to the genus *Trifolium* in Türkiye, together with the species reported in this study, is 104.

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Research article

Evaluation of products grown with pesticides in terms of Islamic law

Ali Yuksek*[1]

[1] *Ondokuz Mayis University, Faculty of Divinity, Department of Basic Islamic Sciences, 55200, Samsun, Türkiye*

Abstract

Throughout history, healthy food has been one of the most important issues of every society. Religious, halal, clean and healthy food perspectives are the most natural rights of every person and it is necessary to respect others right. A Muslim must be sensitive and aware about halal and haram in their lives and one of the most important issues related to halal and haram is food. A wide variety of foods are consumed every day. However, the food is obtained due to biotechnology tools which are part of our daily lives. Firstly, the safety of these products in terms of health, and secondly is whether these products are halal for Muslims. Indeed, protecting human and environmental health is one of religious and humanitarian duty. In this study, it is tried to present the effects of chemical fertilizers and pesticides used in agricultural food production on human, animal and environment and the perspective of the religion of Islam against these effects. These substances have a number of positive and negative effects on people and the environment. Currently, we can see variety of foods as seen natural, contain many harmful substances that threaten human health using technologic tools. At the beginning of the study, the nature of pesticides and how they can affect plants, animals and humans was discussed. In the later parts of the study, it is tried to interpret the halal and haram of these products in terms of Islam.

Keywords: *Chemical fertilizer; human health; humanitarian; legal theory; pesticide*

1. Introduction

Islam religion is the body of divine orders and prohibitions sent by Allah to enable people to find happiness in this life and the hereafter. Protecting the life, property, mental and physical health of people in this integrity is among its main goals (Yuksek, 2018; Baqarah 2/195). It has always recommended what is good and beneficial in all matters and warned people against the bad and harmful. If something is prohibited in Islam, that thing is definitely harmful to people in one way or another. It has many recommendations and imperatives for a healthy and happy life (Kasani, 1982; Cayiroglu, 2014). For example, while Islam stands in favor of cleanliness and health, it has included these among the basic conditions of worship. It has also ordered to consume halal and healthy food for mental and physical health and a dignified life. Therefore, it is forbidden to consume certain plants and animal meats and to drink some beverages (Zeydan, 1976; Yuksek, 2016).

It was easy to know about prohibited and suspicious foods in the past. These are listed in fiqh books (Ibn Rushd, 1975; Kasani, 1982; Zuhayli, 1991). However, today, the widespread use of biotech foods and production of products, whose ingredient can only be known by those concerned, have made this (Yaradoddi et al., 2024). This has become even more difficult especially with the introduction of chemical/synthetic pesticides, genetically modified products and chemical fertilizers in agricultural production (Tokel et al., 2022; Hutsaliuk et al., 2024). In the past, the lack of color, taste and odor of water, the natural color and taste of a foodstuff, or the absence of a prohibitive religious text about a product were sufficient for its consumption (Kasani, 1982; Zuhayli, 1991; Taberi, 1998). Lately, we can see that many natural looking fruits and vegetables can actually be full of harmful substances that threaten human health. We started looking at almost every product with suspicious eyes (Sharma, 2015; Sharma et al., 2018). Scientists have a great responsibility to ensure that the

* Corresponding author.
E-mail address: ali.yuksek@omu.edu.tr (A. Yuksek).
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products we consume are healthy and halal. It is of great importance that scientists work meticulously and to warn people and to provide people with the right suggestions when necessary in order for people to reach healthy and halal food (Yuksek, 2018). As far as we know, no provision regarding the interpretation of the products obtained with the chemicals used in agriculture from the Islamic point of view has been found in the literature. Therefore, the purpose of this research is to discuss the suitability of the products obtained from the agricultural areas, where chemical substances are used in recent years, from the Islamic point of view.

2. Method

2.1. The purpose and importance of the research

One of the most important problems of Muslims today is access to halal and healthy food. Deep concerns have been raised about the food being healthy and halal, especially along with the production of chemical pesticides and fertilizers and genetically modified products after the green revolution in agriculture. This study points out the elements that threaten our health to some extent. There are various studies on that subject (Dhankhar and Kumar, 2023; Kaur et al., 2024; Yadav et al., 2024). However, there are very few studies that include the evaluation of these products from the Islamic point of view (Medani et al., 2024). It was aimed to contribute to this field with this article.

2.2. Hypotheses of the research

Our world today is faced with a number of problems. The most important of these are the factors that threaten human and environmental health. Most of these problems are produced by people due to various concerns. Some of these are pesticides and chemical fertilizers, which are widely used against harmful organisms seen in agricultural production areas and are harmful to the environment and human health. This situation also adversely affects the daily life of the Muslim, because it is necessary to consume halal food for belief and worship life. Scientists have a great responsibility in the matters of providing healthy and halal food and warning people against pests.

2.3. Scope and limitation

The scope of our research consists of presenting the positive and negative aspects of chemical fertilizers and chemical fertilizers and their varieties used in agriculture with scientific criteria, and then evaluating them in terms of Islamic Law.

2.4. Methodology

This research is an interdisciplinary joint study. The results were achieved by blending the subjects of medicine, biology, agriculture, chemical pharmacology and genetic science data within the framework of Islamic texts.

3. Findings and interpretation

3.1. Pesticides and their types

Chemical or biological control substances used during the production, consumption and storage of food products in agricultural production in order to destroy the living creatures or to reduce their damages that reduce the mineral-vitamin value of the products and cause damages to products are called pesticides (Stephenson et al., 2006; Zacharia, 2011; Botitsi et al., 2017). Pesticides, in other words, are all kinds of chemicals and natural substances used in agricultural control. These are chemical substances consisting of various chemicals or their mixtures used to prevent harmful organisms such as insects and disease factors seen especially in agricultural production areas, and to reduce the damage of weeds (Nicolopoulou-Stamati, 2016; Meftaul et al., 2020).

Pesticides can be chemical substances as well as microbial creatures like bacteria, (Aksoy et al., 2017), fungus, virus (Bahaman et al., 2020), etc. that can be used as biological agents. Some organisms that damage agricultural products and make pesticide use necessary; disease-spreading insects, weeds, birds, some mammals, pathogens, worms and some microorganisms that damage crops in agricultural production (Gun and Aytac, 2019). Although pesticides have some benefits, they can also pose significant problems for some plants and animals due to their toxicity. These will be discussed in the following sections.

We can give biopreparations consumed for pesticide purposes, insect and plant growth regulators, pheromones, nutritional inhibitors, insect repellents, various traps, plant activators, preparations used in the treatment of physiological diseases as the examples of the chemicals used for agricultural purposes and pesticide-like substances. Pesticides are named according to their intended use. Their types are as follows:

Insecticide: Chemicals used against animal organisms such as insects.

Fungicide: Chemical substances used against fungi.

Herbicide: Chemicals used against weeds.

Molluscicide: Chemical substances used against animals that are classified as mollusks.

Rodenticide: Chemicals used against rodents.

Nematicide: Chemicals used against nematodes.

Acaricide: Chemicals used against mites (Chiari et al., 2017; Derbalah et al., 2019; Bertero et al., 2020; Ebadollahi et al., 2021; Kankam, 2021; Wang et al., 2024).

Pesticides have wide range of uses in agricultural production, especially against pests, diseases and weeds. At the same time, it is continuously used in herbal and animal production against internal and external parasites. Due to this wide range of use, pesticides contain many different auxiliary substances in addition to many active substances in their formulations. For this reason, pesticides can create very different effects from each other (Tiryaki et al., 2010; Yadav and Devi, 2017; Ansari et al., 2024).

3.2. History and usage stories of pesticides

Pesticides used in agricultural production have been used at primitive level since the formation of settled civilizations. However, with the green revolution in agriculture, it has been widely used in almost every part of the world. The use of pesticides has become widespread, especially in order to increase productivity in agriculture and to combat pests that prevent it (Arvas and Kaya, 2019; Mansfield et al., 2024).

The first pesticide used is fungicide. The substance used for this purpose is sulfur. Later, it was used as an insecticide in materials such as arsenic, copper and iron. These are inorganic substances. The first natural organic compounds used were

derris, nicotine and pyrethrum (Banaszkiewicz, 2010; Dagar, P., and Ramakrishna, 2024).

The variety of chemicals called pesticides has increased rapidly after 1950. This increase also resulted an increase in its usage rates. DDT, which is banned in Turkey and Europe due to its health hazards, is a pesticide that is classified as the first synthetic organic insecticide used. Later, pesticides such as 2,4-D and MCPA, which are still classified as herbicides, have emerged commercially (Yadav and Devi, 2017).

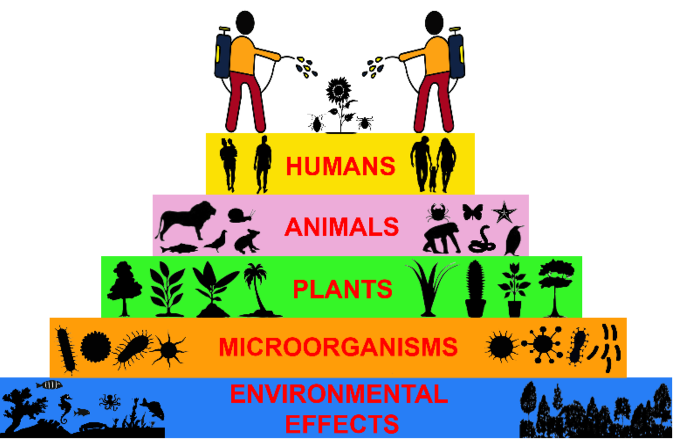
In 2019, the agrochemical market worldwide was worth approximately 234.2 billion U.S. dollars. This is expected to increase to more than 300 billion U.S. dollars in 2025 (Statista, 2010). The most important pesticides produced and consumed are insecticides, fungicides and herbicides respectively. In the world, herbicides rank first among pesticides with a share of 47%; it is followed by insecticides with 29% and fungicides with a share of 19%. Herbicides and insecticides make up more than 70% of the pesticides consumed. Pesticides consumption varies by region and country. For example, while it is 15.3 kg/ha in Columbia, 3.10 kg/ha in China, 8.8 kg/ha in Netherlands and 2.2 kg/ha in the USA (Plummer, 2013); whereas it is 1.3 kg/ha in Turkey, which is lower when compared with the other countries (Ozdem and Karahan, 2018).

3.3. Toxicity of chemical pesticides

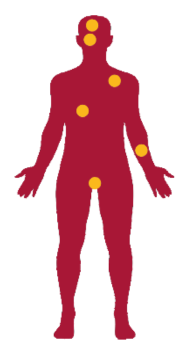
The fight made by using chemicals with natural and/or synthetic properties that have the effect of killing unwanted living creatures that cause losses in plant production is called "Chemical Control" (White and Leesch, 2018). Pesticides, which are chemical control agents, kill the unwanted organism due to reasons such as blocking the nervous system, blocking a vital protein synthesis, disrupting the nervous system, damaging the skin, and destroying digestive organs of the living creatures (Huong et al., 2020; Lee et al., 2020; Prakash and Verma, 2020). The toxic effects of pesticides can be sudden or gradual. This depends on the active ingredient of the pesticide, as well as on its amount. The effect of pesticides weakens relatively over time. At the same time, pesticides can harm non-target beneficial organisms. In addition to these, pesticides released into the nature damage the natural structure and can enter the food chain as residue (Cozma et al., 2017; Kadlikova et al., 2021).

Synthetic chemicals that enter the body structure of living creatures are affected by bio-chemical reactions in the body of the living creature and over time they break down and turn into less harmful/harmless forms, and/or toxic metabolites. Chemicals that get into the body of the unwanted plants, poultry creatures that cause harm and the microorganisms that cause disease, are mixed into the nature from the body of the living creatures as a result of the killing effect and are included in the passive decomposition process (Abdulrazaq et al., 2020; Erguc et al., 2021).

Pesticides that are mixed with nature have a tendency to decompose and decrease their damage relatively due to the effects of soil, water, sun and living creatures. As a result of the aforementioned decomposition, some of the new forms may be harmful, accumulate in the earth or in the bodies of living creatures, but eventually decompose over time in nature (Yadav and Devi, 2017; Ozdem and Karahan, 2018). Affected systems, acute and chronic mode of actions of pesticides are explained in general in Fig (Mackley-Ward, 2022). For example, the Dalapon herbicide causes the plant to die because it stops the production of pentanoic acid (Kaya et al., 2013; 2020).



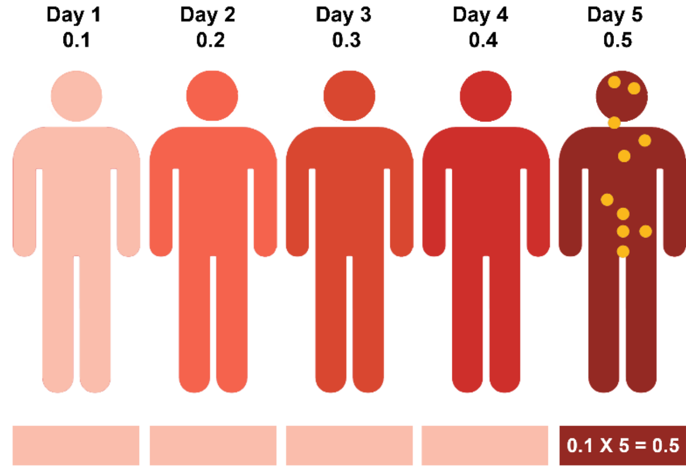
Affected Systems



Acute Symptoms

Acute Symptoms: Endocrine disruption, microbiome disruption, inheritable genetic effects, neurological issues, mental illness and cognitive impairment, liver damage, lung and respiratory tract illness, immunological issues, carcinogenic effects, reproductive issues and developmental issues.

Chronic Symptoms: Nausea, vomiting, abdominal cramps, skin and eye damage, twitching, weakness, tremor, headaches, dizziness, slurring, seizures, lung and respiratory irritation.



Chronic Symptoms

Fig. Systems affected by pesticides, and their acute and chronic effects on humans.

It is a known fact that the use of these pesticides, which have the effect of harming and killing unwanted living creatures, in agricultural production, they can cause many damages and pose risks for soil, air, humans and animals besides its some benefits. For this reason, these chemicals, which are referred as agricultural poison and called pesticides in the literature, are generally produced with advanced technologies and are released

to the market after being subjected to controls. The evaluation of pesticides is made in terms of biosafety and it is aimed only to harm the target organism and not to harm other living creatures and nature (Pagano et al., 2023; Asefa et al., 2024; Oirdi et al., 2024). The production and consumption processes of plant protection products are implemented according to a number of important standards developed by the International Plant Protection Convention (IPPC), which operates under the United Nations Food and Agriculture Organization (FAO). These standards are easily accessible on many websites (IPPC, 2021; FAO, 2021)

In addition, there are a total of 341 Standards on Plant Protection Products created by the European and Mediterranean Plant Protection Organization (EPPO), of which Turkey is a member, based on geographical and regional variations and developed with the participation of all relevant experts in the relevant field and updated frequently according to the needs. Again, these standards can be accessed from the internet addresses (EPPO, 2021).

4. Islamic literature

Since the subject we are working on involves two different fields, we consider it appropriate to include concepts such as pesticides, fungicides, and toxic, and then some Islamic concepts such as haram (unlawful, forbidden), halal (lawful or allowed, permissible) and istihalah (change of form, transformation) for the experts of both segments to understand the subject well. Thus, we think that the conclusion part will be better grasped.

Halal: It means free, permissible in the dictionary and not prohibited in terms of Islam (Feyrûzâbâdî, 1987; Erdogan, 1998). As the Islamic term, halal is allowed by Allah to be done and the prohibition and sin knot has been resolved (Karadavi, 1973), which is religiously permissible to do, a deed that is permissible, there is no question in its execution or use according to Sharia Law (Koca, 1998; Diyanet İşleri Başkanlığı, 2006). The words Mubah, Caiz and legitimate are used in the same sense as the word halal. It is the opposite of haram (Diyanet İşleri Başkanlığı, 2006). There are many different variants in Quran and Sunnah (Baqarah 2 / 296,228,229,275; A'raf 7/157; Tahrîm 66/11; Maidah 5 / 5.88; Nahl 16/116; Hajj 22/30; Taha 20/27; Maidah 5/2 Baqarah 2/275; A 'raf 7/157; Tahrîm 66/11; Abu Dawud, "Tahâret" 41; Tirmidhi, "Tahâret", 52; Bukhari, "Adhan", 8; Muslim, "Salat", 11).

Haram: It means something that is forbidden in the dictionary or something that is forbidden to a person, not permissible and not halal (Feyrûzâbâdî, 1987). As an Islamic term, haram refers to a deed that is strictly and bindingly required not to be committed (Koca, 1997; Kocak et al., 2015).

This certainty and bindingness are due to the fact that the request reaches us with conclusive evidence, that is, with mutawatir evidence. In order for a deed or something to be haram, it must be prohibited by both the verses of the Qur'an, and the evidences that are definite or almost definite and whose intention is clearly understandable, such as mutawatir or the famous sunnah (Diyanet İşleri Başkanlığı, 2006). To commit haram is a sin, it entails blame. A person who commits haram will suffer torment in the hereafter, and there may be some religious punishments in this world. Acts such as eating pork, consuming alcoholic beverages, backbiting, talebearing, and committing adultery can be examples of haram. Haram things may cause material or moral harm to humans, animals and the environment. But harm in an absolute sense does not mean haram. Sometimes Allah may prohibit things just as a test. A person who commits haram must repent. Considering something forbidden by Islam as haram is a matter of faith. Accordingly, it is blasphemy to deny something that is forbidden with definitive evidence (Diyanet İşleri Başkanlığı, 2006; Atar, 2011). The authority to make something haram or halal belongs to Allah in the absolute sense, and with his permission, the Prophet can also make something forbidden (Yunus, 12/40; Nisa 4/80; Ahzab, 33/36; Nisa, 4/59; Taberi, 1998; Kocak et al., 2015).

Tayyib: It means the opposite of filth, something that is not dirty/filthy; it means clean, permissible/halal, pleasing (Yerinde, 2011). In addition, clean foods that do not harm the mind and body, and the things that do not have the rights of others are also called tayyib (Erdogan, 1998). Tayyib is also used in the Qur'an to mean halal and clean foods, halal meat, plants, fishes and spoils, people who protect themselves from ignorance and evil, clean lands, righteous deeds (Taberi, 1998; Senol, 2014).

Habis: Lexical definition of habis (foul)is the attribute of everything that is foul, whether in terms of taste or smell; it is defined as something that is tangible or abstract, which is disliked because of its essence, being dirty, disgusting and worthless (Duman, 1996). According to the Quran, when a food is habis, it means something haram. Habis foods are harmful both materially and morally (Senol, 2014).

Najasat: In the dictionary, it is the opposite of taharet, that is, cleanliness. It means material or spiritual dirt/filth. As an Islamic term, najasat is impurities such as blood, swine, mayta, which have religious evidence that they are dirty [Mâide 5/3 "It is forbidden to you to eat mayta (dead animal meat), blood, pork and animals slaughtered in the name of anybody other than Allah"]. What is essential in Islam is that the substances are clean. Impurities have been classified in various respects. These are as follows:

Necaset-i Galîza (Heavy filth): Blood, urine, stool, semen, pieces of meat severed from the body, pus oozing from wounds, purulence, vomit and pork meat and blood, etc. are dirt/filth. If they exceed a certain area in the human body, clothing or place of prayer, they will prevent the acceptance of pray. In addition, animal carcasses that are not slaughtered according to the Islamic method or died by themselves, and the feces, urine and saliva of animals, whose meat is not eaten, and the feces of some animals such as goose and chicken, whose meat is eaten, and the flowing blood of land animals, wine and all alcohol-containing beverages according to the preferred belief are najasets (Yuksek, 2018).

Najaset-i Hafife (Light filth): These are filths, for which there is no definite evidence or for which there is a dispute about being filthy according to sharia. We can give examples of the feces and urine of animals such as horses, mules and donkeys, and sheep, goats, and cattle whose meat is eaten (Baysa, 2018).

Everything is clean according to sharia as long as there is no evidence to suggest that something is filthy, impure (Mecelle, Article 5). According to this, the soil types of the earth, solid-liquid mines extracted from underground, water, all plants and their fruits are clean. Fruit and vegetable juices, vinegars are clean. The human body is clean as long as it does not contain impure material. The water that remains from wudu and ghusl is pure and clean according to sharia. Fish species and dead fish considered as clean. The blood remaining in the flesh of animals slaughtered in accordance with Islamic methods is also considered clean. The external bodies of animals are clean. Pig

is an exception to this. The solid parts of the dead animal such as bones, horns, feathers and teeth are clean. If human and animal feces are burned and turned into ash, they are also clean (Diyanet İşleri Başkanlığı, 2006).

Istihalah: In the dictionary, it means the change and transformation of the essence of something (Feyrûzâbâdî, 1987; Cevheri, 1984; Erdogan, 1998). There is a change in the nature and quality of something (Feyyumi, 1987). Istihalah as an Islamic term means the transformation of one nature into another, the transformation of something from its own nature and quality into another nature and quality, the change of impure substance by itself or by a means and to become clean (Zuhayli, 1991). In other words, it can be defined as the transformation of the nature/essence of something, which is impure or haram to use, from itself into a completely different substance in terms of names, attributes and features (Erdogan, 1998). Istihalah can occur in different ways. It can be through combustion, fermentation, solidification, having life, chemical processes, etc. (Cayiroglu, 2014; Aslan, 2016). For example, the transformation of wine into vinegar without adding any additives into the wine, and the burning of animal excrement into ash is such a process. Kasani (1982), one of the Hanafi scholars, state that the soil changes the impure substances over time, in other words, it turns it into its own nature by causing metamorphosis. Therefore, we can say that istihalah is the transformation of a substance into another substance that is completely different from itself (Yuksek, 2018). Today, istihalah concerns many areas such as the food, cosmetics and pharmaceutical industries. Scientists discuss whether chemical fertilizers and pesticides are clean, healthy and halal after being absorbed and transformed by plants. This subject is also one of the important issues of our study.

Istihlak: Basically, it means extinction, perishing, ending something (Mustafa, 1972). As a term, it means a small amount of haram or impure substance mixing with a large amount of halal and clean substance and as it spreads and disperses in it, although it continues to exist, it loses its features such as color, taste, smell. For example, a small amount of alcohol or blood, which is considered impure, mixes with water or another liquid, and disappears inside it. Alcohol and blood in this situation are considered as consumed. Even though they do not change the taste, odor and color of the liquid they mix into, its presence there is obvious. Although istihalah is a chemical change, istihlak is a physical event (Cayiroglu, 2014; Yuksek, 2018).

4.1. Basic criteria for the permissibility and prohibition of a product in Islamic law

In Islam, the primary principle is permissibility and freedom. Prohibitions are limited in number. There are many factors that may lead to something being prohibited in Islam. These factors can make a food item or action prohibited. We can summarize these factors briefly as follows:

4.1.1. The textual evidence factors

Textual evidence in Islamic literature refers to the Quran and hadith texts (Erdogan, 1998). Whether something is halal (permissible) or haram (prohibited) is determined primarily by the presence of clear textual evidence. Ultimately, the authority to determine what is halal and haram belongs to Allah. In this regard, Allah says: “Do not say about what your lying tongues describe”: “This is lawful and this is forbidden”, to fabricate lies and attribute them to Allah. “Those who fabricate lies against Allah will not prosper” (Nahl, 16/116). Therefore, whether something is considered halal or haram, or whether a food item is permissible to eat, is within the decree of Allah. With Allah’s permission, Prophet Muhammad may prohibit or permit something. As mentioned in the Quran: “Whoever obeys the Messenger has obeyed Allah” (Nisa, 4/80); “Whatever the Messenger gives you, accept it, and whatever he forbids you, refrain from it. Be conscious of Allah. Allah is severe in retribution” (Hashr, 59/7). In Islamic jurisprudence, the principle is that “Where there are Quran and Sunnah texts, there is no room for personal reasoning (ijtihad)” (Majalla, 1876, 14). Therefore, a Muslim servant obeys or refrains from something solely because Allah commanded or prohibited it, seeking to earn His pleasure. The servant focuses on fulfilling Allah’s command rather than analyzing the benefits and wisdom behind it. A believer readily accepts and stays away from many prohibitions stated in the Quran without questioning them, as part of their devotion (Erdogan, 1998). For example, the requirement to mention the name of Allah when slaughtering an animal is a commandment of this nature. This practice has no direct impact on food safety (An’am, 6/121; Haddad, 1978; Yuksek, 2018).

4.1.2. Benefit and harm factor

Harm is the opposite of benefit and profit, describing the material or spiritual loss, poverty, hardship, illness, danger, and other negative aspects that an individual face. Harm is characterized as a decrease in material or spiritual wealth without one’s consent (Erdogan, 1998). These meanings are frequently used in various verses of the Quran and the words of the Prophet (Fath, 48/12; Baqara, 2/102; Muwatta, Akziye, 31). Losses in an individual’s material or spiritual values, hardships, bodily harm, or deteriorating health are considered harmful. In other words, harm refers to subsequent losses or damages in an individual’s spiritual, physical, or financial well-being. Harm is categorized into types based on whether it is general, severe, material, or spiritual (Aybakan, 2013).

According to the teachings of the Prophet, a Muslim cannot harm others and cannot be content with harm inflicted upon themselves (Muwatta, “Akziye”, 31; Ibn Majah, “Ahkam”, 17). Providing ease, eliminating hardship and harm, and considering the general and specific benefits of individuals are the fundamental goals in Islam. Therefore, rules have been established in Islamic law regarding the prohibition of actions and behaviors that cause harm and compensating for the harms endured. In principle, necessary measures should be taken to prevent harm, importance should be given to precaution, legal rights should be protected when harm is incurred, and patience should be shown in the face of incurred losses. Despite efforts, it is not entirely possible to be immune to harm in this world. Hence, when evaluating an action or product, consideration is given to whether the benefit or harm outweighs the other. Based on this evaluation, religious judgments are made (Sener, 1970).

In regulations related to harm, a two-fold objective is pursued. The fundamental aim is to prevent the occurrence of harm. In such regulatory frameworks, preventive measures are considered akin to preventive medicine, starting from the principle of precaution. In cases where it is impossible to completely prevent harm, efforts are made to prevent the exacerbation of harm and to minimize it as much as possible. The regulations in the first phase are for preventive purposes,

while those in the second phase are for compensation.

Preventing harm is more important than providing benefit (Mecelle, Article 24). Sometimes, when a legitimate behavior is used for an illegitimate result, that behavior is prevented (Mecelle, Article 24; Cabir, 2011). Harm is prevented. What causes harm is prevented. The person who caused the damage is compensated for the damage (Aybakan, 2013; Kahveci, 2002).

One of the reasons why some foods and beverages or the chemicals used in their cultivation are haram is that the substance is harmful to human, animal or environmental health. Because the main purpose of all the provisions in the sacred texts is to realize the five basic purposes of Islam. These are to protect the mind, religion, life, property and generation (Kocak et al, 2015). For these reasons, poisonous mind-blowing substances that harm human mental and physical health have been banned. This is also the reason why it is forbidden to eat stinky and spoiled foods. Because Allah wants people to eat halal and clean things (A'raf 7/157). Physical and mental health enables people to live a happy, prosperous life. In addition, a person is obliged to protect the mental and physical health entrusted to him. It is a requirement of servitude to stay away from things that will harm him. One of the most basic conditions for being able to serve Allah is to have a healthy mind and body (Assaf, 1999; Kahraman 2012).

4.1.3. The prevalence of harm over benefit

In Islam, if something has both benefits and harms but the harm outweighs the benefit, then that thing is prohibited. A prime example of this is alcoholic beverages. In this regard, Allah says in the Quran, "They ask you about wine and gambling. Say, in them is great sin and [yet, some] benefit for people. But their sin is greater than their benefit" (Baqara 2/219). While Allah mentions the benefits of alcoholic beverages in the Quran, He has prohibited them because their harms outweigh the benefits. The Prophet also described alcohol as the mother of all evils (Ibn Majah, Ashriba, 1).

4.1.4. Something being harmful to humans, animals, or the environment

Islam has allowed things that are beneficial to all creatures and prohibited things that are harmful. Humans are responsible for preserving their bodies and their health, which are entrusted to them (Assaf, 1999). One of the fundamental goals of Islam is to protect the health of the mind, body, and environment (Cayiroglu, 2014). Anything that contradicts this goal should be prevented. Therefore, anything that harms an individual's mental and physical health is considered prohibited in Islam. The prohibition of harmful substances like drugs, poisons, and intoxicants is based on this principle (Assaf, 1999). The main reason why drugs like heroin, marijuana, opium, and cocaine are prohibited is that they harm an individual's mind, body, lineage, religion, and reputation (Zerkesi, 1990). In this regard, Allah has commanded, "Do not put yourselves in danger with your own hands..." (Baqara 2/195); "Do not kill yourselves" (Nisa 4/29).

4.1.5. The perception of something being repulsive or disgusting to a sound mind

Whether something is considered good or repulsive is dependent on the perspective of a sound mind. For example, a sound-minded individual would not find blood, carrion, mice, snakes, scorpions, flies, spiders, pus, semen, pre-ejaculate, or urine appealing (Serahsi, 1989; Cessas, 1994; Kasani, 1982; Ibn Nujeim, 1997).

Each individual's preferences and tastes can vary. A person's morality and character are shaped according to the region and culture they live in. Therefore, a type of food that a person enjoys may be considered halal (permissible) only for them, while consuming something that their soul finds repulsive could be haram (forbidden) for that person alone (Şa'rânî, 1989). Thus, when there is no evidence to determine whether something is halal or haram, it is appropriate to act according to scientific evidence and sound reasoning (Riza, 1946).

4.1.6. The consumption of meat from predatory and wild animals

Animals such as lions, tigers, wolves, jackals, hyenas, bears, and monkeys, as well as predatory birds like eagles and vultures, which are aggressive, catch their prey with claws and canine teeth, kill, tear apart, and generally feed on meat, are referred to as predatory wild animals. Prophet Muhammad prohibited the consumption of meat from these animals (Abu Dawud, Et'ime, 33; Leknevi, 2009; Cayiroglu, 2014). The wisdom behind the prohibition is that predatory animals usually feed on carrion and impure substances, which can potentially transmit diseases to humans.

4.1.7. Acceptance of something as impure based on custom

In matters where there is no ruling in the Quran or Sunnah, Islamic scholars commonly refer to the consensus or analogy of scholars (Yunus, 10/71; Kocak, et al, 2015). If there is no clear evidence, Islamic scholars unanimously agree that customs and traditions can be considered (Baqara, 2/233; A'raf, 7/199; Zeydan, 1976). Some words, actions, or food items are considered haram based on customs, and religious rulings are made accordingly. Therefore, societal perspectives are essential in the prohibition of certain things.

4.1.8. The manner in which a product is obtained

Another reason a product may be considered haram is if it is obtained without adhering to Islamic principles. For example, eating an apple is halal, but consuming someone else's apple without permission is forbidden in Islam. Forbidden items are categorized based on their essence or how they are obtained. For instance, an animal that dies on its own, pork, or wine are explicitly forbidden in the Quran. However, if a product or a food item is obtained through fraud, deception, theft, gambling, bribery, or interest, then these are also considered within the scope of haram (forbidden) (Kocak et al., 2015; Cayiroglu, 2014).

4.2. The importance of health in Islam and other religions

Protection of individual and public health is one of the main goals of the Islam religion (Kocak et al., 2015). Therefore, it attached great importance to the mental and physical health of people. It prohibited any food, drink, behavior or life that would destroy or restrict them. It has made some legal arrangements for their protection. For example, Allah Almighty in the Quran emphasized this with his verses "Let not your own hands contribute to your destruction" (Baqarah 2/195); "He makes the

clean things lawful (halal) to them and prohibits (haram) all corrupt things" (Araf 7/157). The Messenger Muhammad (SAW) also pointed out the importance of the matter by saying:

Islam has laid down important principles for the protection of health. For example, it prohibited murder and causing death (Nisa 4/93), endangering health and life (Baqarah 2/195), consuming food, drink and harmful substances that deteriorate mental and physical health of the human being such as alcoholic beverages and drugs (Maidah 5/90), blood, carrion, pork and their products (Baqarah 2/173). As a matter of fact, the wisdom in the prohibition of these harmful substances is the protection of human health.

Again, it is not permissible to use various toxic substances, bleach, vitriol (nitric acid), harmful chemicals, unnecessary drugs used for various purposes that harm the mental and physical health of the human being. This prohibition is again expressed on the verses of Allah "Do not kill your own souls" (Nisa 4/29); "Do not endanger yourself with your own hands" (Baqarah 2/195) (Boran, 2016).

Here, when we look at the holy texts of Islam on eliminating health and endangering it, it is difficult to view the use of pesticides and yield-increasing drugs in agriculture as religiously permissible, which have scientific data today which indicate that they adversely affect our health and are harmful (Blair et al., 2015). As a matter of fact, it is one of the basic principles in Islam that everything that causes harm is prohibited and that what causes harm must be removed immediately. There is a consensus among Islamic scholars that it is not permissible to produce and consume harmful substances (Aybakan, 2013; Kocak et al., 2015).

In addition, the fact that Islam attaches importance to cleanliness, hygiene, healthy and halal food and accepts them as faith (Müddessir 7/4), the requirement of cleanliness in prayers, washing hands before and after meals, and brushing our teeth are always underlying the value Islam attaches to health and its attitude towards protecting it.

4.3. Health in other religions

Protecting health and banishing diseases and what causes them are among the goals of other religions. For example, in the scriptures of Judaism and Christianity, there are many passages on the protection and maintenance of health. For example, in the scriptures of Judaism and Christianity, there are many passages on the protection and maintenance of health. Because people not in good mental and physical health, cannot fulfill their duties towards their Lord and the society (Numbers, 19/11-22; Deuteronomy, 23/12-14; Exodus, 15/25-26; Cantique Rabbah, 6, 11, (9); Luke, 4/31-37; Luke, 4/40-41; Markos, 1/40-44; Luke, 13/10-16, 14/1-4, 22/50-51).

5. Impact of pesticide types on health of living organisms and environment, potential risks and considerations

Environmental impact: Studies on the effects of chemical pesticides used in agriculture in our natural habitats and their interactions in the environment have increased further with the collaboration of toxicologists, microbiologists, agricultural engineers, biologists, chemists, ecotoxicologists, and biotechnologists (Tiryaki et al., 2010; Yadav and Devi, 2017).

Pesticides released in the environment during the production and/or usage phase cause pollution. In the new scientific data indicator, it has been shown that pesticides enter the natural cycle and have harmful effects on the water and the soil, thus the environment, as a result of some pesticides used against aquatic plants being applied directly to the water and soil (Führ, 1982; Rajmohan et al., 2020).

While some of the pesticides can be applied directly on the soil, some of them are applied on the plant or to the seeds as a seed coating. Some of these, especially in pesticides applied to the surfaces of plants, pass to the soil and then to the elements of the other ecosystem, and therefore, pesticides can move in the soil over time depending on the solubility, quality and type of the soil, persistence and climatic factors. As a result of the researches, it has been proven that if some organic chlorinated pesticides are applied to the soil, more than 50% of them can remain in the field for 15-16 years. And thus harm the environment (Wang'ombe, 2014; Rajmohan et al., 2020).

Impact on living organisms: Pesticides that affect all living creatures have a harmful effect on many living things, especially humans. For example, it is seen as harmful on human skin, respiratory tract, digestive system and many other organs as residue. Especially DDT (Dichlorodiphenyltrichloroethane), Hexachlorobenzene (HCB), Endrin, Aldrin and Heptachlor and similar herbicides, organic chlorides, which have long-term persistence in nature and the capacity to accumulate in the organism, also cause chronic poisoning, albeit at low doses (Yildiz et al., 2005; Duke, 2017; Jonathan et al., 2024).

Effects on body and organ weights: Some pesticides affect body and organ weights of humans and some other living things in different scale and periods (Dinca et al., 2023).

After various scientific studies on humans, it has been revealed that pesticides show hematological toxicity effect. For example, some pesticides may affect the hematological parameters and the leukocyte system in non-identical manner (Lee et al., 2016; El-Gendy et al., 2022).

Immunotoxic effects: Synthetic chemical pesticides such as PCBs, chlorinated dibenzo-p-dioxins, endosulfan, aldicarb, carbaryl, carbofuran, malation, atrazine and 2,4-D have significant immunotoxic effects. These substances increase the risk of autoimmune reactions and cause an increase in allergic reactions (Fujitani et al., 1997; Kim et al., 2017).

Dermal effects: Chemical pesticides such as Benomyl, DDT, endosulfan, which were frequently used in the past, caused contact allergic dermatitis in people working in agriculture (Dennis et al., 2010; Ambaye et al., 2024).

Biochemical effects: Pesticides used for agricultural purposes progress to the living body via the skin, respiratory or digestive organs and are metabolized in the liver by the cytochrome P450-dependent monooxygenase system and cause side effects (Bailey et al., 2015; Shinya et al., 2023).

Teratogenic and mutagenic effects: According to the latest findings, it has been proven that pesticides have teratogenic and mutagenic effects. The presence of dose and stress is an important factor in the creation of the teratogenic effect. Pesticides with teratogenic effect are 2,4,5-T and 2,4-D herbicides, organic phosphorous, captan, folpet and difolatan. Pesticides with mutagenic effects (diazinon, ziram etc.) can cause chromosomal breakages (Koutros et al., 2009; 2016; Guerrero Ramírez et al., 2023).

Genotoxic effects: a correlation has been found between chemical pesticides, which are almost an integral part of agricultural production in the last century, and cancer incidence. Cancer incidence is generally low among those employed in agricultural production. Nevertheless, in the last quarter century, the risk of specific cancer types increased further among those

working in agriculture (Valcke et al., 2017). These specific types of cancer are leukemia, Hodkin's disease, non-Hodkin's lymphoma, multiple myeloma, and lip, stomach, prostate, brain and breast cancers (Packard et al., 2019; Latifovic et al., 2020; Togawa et al., 2021). In addition, the study of Mehrpour et al. (2014) showed that pesticides containing organophosphorus reduce sperm activity, decrease testicular weight, damage sperm DNA and create abnormal sperm morphologies. Also, pesticides can trigger hypospadias disease (Michalakis et al., 2014).

6. Alternatives to synthetic pesticides

Natural pesticides: From past to present, natural pesticides can be used at different proportions and purposes as an alternative to synthetic pesticides (Khalili et al., 2019; Kocacaliskan et al., 2019). Naturally derived pesticides are the most environmentally friendly alternatives to synthetics, with their low natural persistence and broad biological activity spectrum. In addition to this, these products of natural origin have few side effects on other living creatures, and the development of these natural products continues with computerized bioinformatics tools (Bahaman et al., 2020). Some herbal extracts have high toxicity and repellent effects against pests, common dandelion/*Cinnamomum camphora*, trigonella and cinnamon can be given as an example to these (Roy et al., 2015; Abdul et al., 2019; Kaya et al., 2019).

Microbial pesticides: Importance of the microorganisms that have been used consciously and unconsciously from the past to the present have increased in recent years. Microorganisms have been used as natural pesticides in recent years (Aksoy et al., 2018). Thus, the damages of some pesticides have been tried to be eliminated in a natural way (Sadof and Sclar, 2000; Dayakar et al., 2015; Akcay and Kaya, 2019).

Non-chemical weed control: The important thing in this method is to prepare the soil well before growing the crop. For example, it is important to make soil analyzes beforehand and to use appropriate soil improvement methods. These will minimize the effects of unwanted plants and organisms. In addition to crop rotation in agricultural production, appropriate soil tillage and mechanical interventions are methods that can be an alternative to chemical weed control in certain areas (Pannacci et al., 2017; Mennan et al., 2020).

7. Scientific consensus on use of chemicals

One of the principles of increasing productivity in plant production is to eliminate harmful organisms from the plant. In the improvement studies carried out in herbal production, no new findings could be obtained that would lead to a significant increase in yield in economically important plants such as wheat, corn, rice, cotton, sunflower etc. since the beginning of 2000s. In other words, the possibility of increasing the total agricultural production through improvement has become very low and the genetic capacity of the species has almost reached the limit. In addition, some important negative effects of the agricultural production model made in the last two centuries on natural resources, especially soil, water and biological diversity, have become visible (Aksoy et al., 2021; Tokel and Erkencioglu, 2021; Tokel et al., 2022).

The 21st century prediction for 9 basic factors that will affect agricultural production is given below. On a global basis, increase of 30% (9-10 billion) in the human population, 60% in food need, 160% in greenhouse gas emissions, 4°C in global warming, and increase in disease and pest pressure are predicted. However, it is calculated that there will be a decrease of 23% in vegetative biological diversity, 24% in the land size per person, 20% in water used in agriculture, and 8% in yield per unit area. It would be more correct not to consider phytosanitary studies only as reducing losses in primary production. Herbal products continue to be damaged by harmful organisms after the fields, vineyards, orchards and greenhouses. Because the failure in taking the necessary measures in plant health may cause conditions, which are harmful to human health, impair food safety and cause food loss of around 15% after production, such as insect infestation, mold and toxin development caused by bacterial or fungal factors. It is calculated that at the end of the 21st century the world population will be 50% more than today and the food demand will be 60-70% more than the present day with the effect of the diet regime. So, when asked what could be done in this situation, the first and only answer to this question is the plan to increase total global food production of today by 60-70% (IPCC, 2021).

While the total population of the world in 1900 was approximately 1.5 billion in 1900, it is more than 7.5 billion today. The United Nations expects the human population to continue to grow and reach 9-10 billion by 2075. The population of Turkey is expected to be 98.1 million in 2055, then 100 million and afterwards stabilize by decreasing a little (United Nations, 2021a).

In the light of the latest scientific information obtained, the human population has increased approximately 4 times in 100 years and it is expected that it will increase by 50% in the next century, but it will stabilize in the 22nd and 23rd centuries (United Nations, 2021b). In addition to these data that affect food production, another issue that should be considered is the daily eating habits. In other words, it is the rate of daily food intake in kilocalories per person. This amount increases day by day with the increasing income level and welfare level. According to the data of the United Nations Food and Agriculture Organization (FAO), the wealthiest 20% of the world population uses 76.6% of the total food produced. Again, 60%, which constitutes the majority of the world population, uses 21.9% of the food produced, while the poorest 20% consume only 1.5% of it. These statistics indicate that the most developed countries (North America and Western Europe) consume 3400-3800 kcal per person, but the rest of the world is only fed by half or less than half of these figures. FAO also stated that the world population suffering from starvation exceeded 840 million by 2020, and also that the population with malnutrition was at least 925 million (World Vision, 2021).

8. Conclusion and suggestions

Preserving the mental and physical health of man is one of the five goals of all divine religions that have been sent down to the world. The religion of Islam attaches great importance to human health for the realization of this purpose. It is against all kinds of factors that will endanger the mental and physical health of the human being. Therefore, any substance that harms him is considered as haram. Islam ordered the consumption of healthy, clean and halal foods, prohibited alcohol and drugs because they intoxicate the mind and negatively affects right thinking, and eating pork, insects, carrion, blood, pus, toxic chemicals because they are harmful to the health of the body.

There is no scientific data showing that the use of natural pesticides is harmful to living things and the environment.

However, according to researches on chemical/synthetic pesticides, it has been demonstrated with scientific data that their residues can be found on plants and living organisms, and that they adversely affect people who consume such foods. Scientific researches have even revealed that these substances are passed on from mother to baby through breastfeeding (Gokmen and Yurttagul, 2001). Therefore, it would not be correct to say that this process is within the scope of the istihalah (transformation) in Islamic law. As a matter of fact, it is a reality that harmful pesticide residues in fruits and vegetables can be seen through technological means. In a study conducted in the UK on infant formulas, the presence of chemical pesticide residues in infant formula was proven, and application made to the court to stop the production of that product. As a result of the lawsuit, the company concerned was found guilty and sentenced. Subsequently, the company's products were withdrawn from the market (Daily Mail, 2021). This situation is important in terms of revealing the danger and seriousness of pesticides.

In addition, the use of pesticides with proven harm to health (mentioned above) to increase product yield or to have some economic benefit cannot make pesticides safe and religiously permissible. Because, Allah commands the consumption of halal, clean and healthy foods and prohibits those who are harmful, useless or those whose harm is more than their benefits. In addition to these, Allah has been a guarantor for the sustenance of every creature He has created.

It is toughed that the production or consumption of products that contain harmful substances are not suitable in terms of Islam. Pesticides with proven negative effects on humans, animals and the environment should be kept at the dosages specified by the relevant experts. Otherwise, it is toughed that it is not suitable for them to be used in terms of Islam.

Ethical statement: During the preparation of this study titled "Evaluation of Products Grown with Pesticides in Terms of Islamic Law", scientific, ethical and quotation principles were observed, the data collected were not tampered with and this study was not sent to any other academic media for evaluation.

Conflict of interest: The author declares that he has no conflict of interests.

Informed consent: The author declares that this manuscript did not involve human or animal participants and informed consent was not collected.

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