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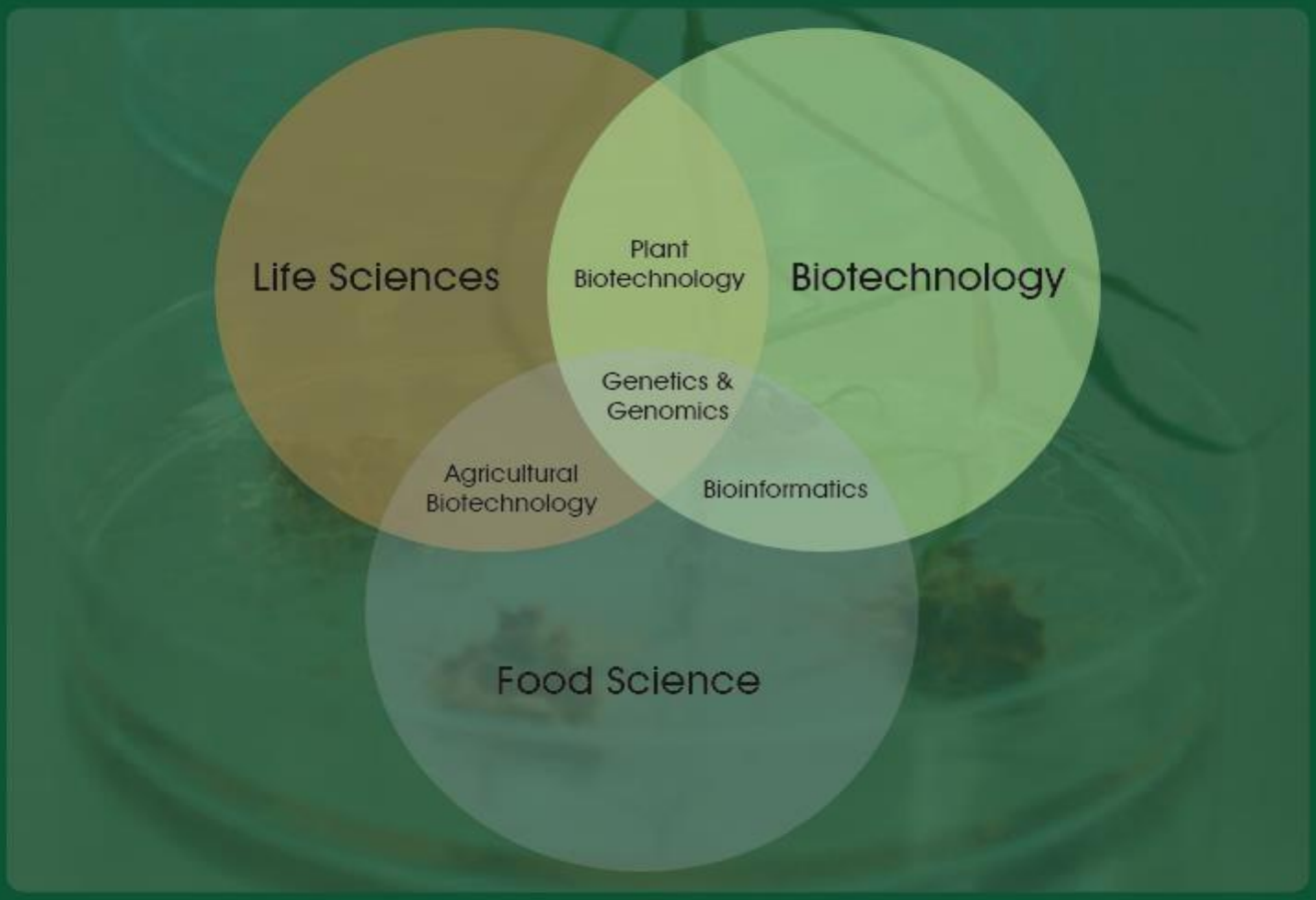

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Yildiz Technical University, Faculty of Chemical and Metallurgical Engineering.
ESENLER/ISTANBUL

Editörden;

Değerli okurlar ve yazarlar,

"International Journal of Life Sciences and Biotechnology" olarak dergimizin on ikinci sayısını yayın hayatına sunmaktan mutluluk ve onur duyuyoruz. "International Journal of Life Sciences and Biotechnology" dergisi araştırma- geliştirme ve uygulama ilkeleri baz alınarak yayınlanan uluslararası hakemli açık erişimli akademik bir elektronik dergidir.

"International Journal of Life Sciences and Biotechnology" dergisi Yaşam Bilimleri, Biyoloji, Biyoteknoloji, Biyomühendislik, Ziraat Bilimleri, Gıda Biyoteknolojisi ve Genetik alanlarındaki ilgili araştırmacılara, kurum ve kuruluşlara teorik ve pratik uygulamalarda katkı sağlamayı, tarafsızlık ve bilim etiği ilkelerine bağlı kalarak çözüm temelli, yenilikçi ve katma değeri olan çalışmalara odaklanan, günceli ve geleceği tartışan çalışmaların yayınlanmasını hedeflemektedir.

Bu düşüncelerle 2022 yılı birinci sayısını yayınladığımız "International Journal of Life Sciences and Biotechnology" dergisini, makaleleri ile onurlandıran akademisyenlere, Fikir / Görüş / Öneri / Katkı ve Eleştirileri ile değerlendirme süreçlerine katkılarından dolayı hakem ve yayın kurullarında yer alan kıymetli bilim insanlarına yürekten teşekkür ediyoruz. Bir sonraki sayıda görüşmek ümidiyle…

15.04. 2022
Editör
Dr. Öğrt. Üyesi Yilmaz KAYA

From The Editor;

Dear Readers and Authors,

As “International Journal of Life Sciences and Biotechnology”, we are pleased and honored to present the 12th issue of the journal. "International Journal of Life Sciences and Biotechnology" is an international double peer-reviewed open access academic journal published on the basis of research- development and code of practice.

The aims of this journal are to contribute in theoretical and practical applications in relevant researchers of Life Sciences, Biology, Biotechnology, Bioengineering, Agricultural Sciences, Food Biotechnology and Genetics institutions and organizations in Turkey, and to publish solution based papers depending on the principle of impartiality and scientific ethics principles, focusing on innovative and added value work, discussing the current and future.

With these thoughts, We are especially thankful to academicians honoring with the articles, valuable scientists involved in editorial boards and reviewers for their contributions to the evaluation processes with through their opinions/ideas/contributions/criticisms in the first issue of 2022 "International Journal of Life Sciences and Biotechnology". Hope to see you in the next issue…

15. 04. 2022

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Assist. Prof. Dr. Yilmaz KAYA

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Research article

Bursal, E., et al., Phenolic content, antioxidant potentials of Saponaria prostrata endemic plant.
International Journal of Life Sciences and Biotechnology, 2022. 5(1): p. 1-8. DOI: 10.38001/ijlsb.989172

Phenolic content, antioxidant potentials of *Saponaria prostrata* endemic plant

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ABSTRACT
Saponaria prostrata (S. prostrata) is an endemic and medicinal plant that contains secondary metabolites such as flavonoids, phenolic compounds, fatty acids, and triterpenoids. This study was carried out to evaluate the antioxidant potentials, and phenolic composition of S. prostrata. Antioxidant properties of the ethanol and water extracts of S. prostrata were evaluated by three different in vitro bioanalytical methods including CUPRAC and FRAP reducing antioxidant methods and DPPH radical scavenging antioxidant method. Effective antioxidant potentials of the plant extracts were found especially in the CUPRAC method. Rutin (36.3 µg/g extract) and hesperidin (32.7 µg/g extract) were characterized as major phenolic compounds of S. prostrata using an advanced HPLC technique.

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Introduction

Traditional medicine is a major part of human healthcare in many parts of the world including developed countries [1]. Due to the resistance and the side effects of the microorganisms against antibiotics, researchers have been focused on plant-derived bioactive compounds used in herbal medicine [2, 3]. The researchers have focussed on plant phytochemicals to find new ways in the treatments of some diseases including cataract treatment [4], cancer metastasis [5], and metabolic inflammation [6]. Some phytochemicals such as hesperidin [7], astragalus polysaccharides [8], and allicin [9] are the key factors in cancer metastasis. Phenolic and flavonoid compounds can be used in the new drug development process[10]. Therefore, the high majority of plants have been recognized as having commercial values [11].

Saponaria prostrata WILLD subsp. *prostrata* (*S. prostrata*) is an endemic and medicinal plant in the flora of Turkey [12] that belongs to *Caryophyllaceae* family [13]. This plant species might be annual, biennial, or perennial plants that branched with

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decumbent prostrate or ascending branches with spreading white hairs [14]. *S. prostrata* contains secondary metabolites such as flavonoids, phenolic compounds, fatty acids, triterpenoids, different saponins, and some other natural sources [15]. These main components are known for their medicinal properties [16]. Phenolic compounds have various biological benefits and have been related to antioxidant properties [17]. Flavonoids have been noted as strength free radical scavengers and demonstrated antioxidant activities [18]. They have preventive effects against the metal chelating properties, hydrogen donor functions, and reducing activities [19]. Due to their natures, green plants, fruits, and vegetables are often being used as primary natural antioxidant sources. The phytochemicals are accepted to reduce the risks of brain dysfunction, cardiovascular diseases, cataracts, and cancers [20]. Compare to the synthetics, consumption of natural antioxidants is better for human health because synthetics have numerous carcinogenic effects. Recently, due to the undesired effects, there is a remarkable request for new and safe natural antioxidants sources [21]. The human body involves both enzymatic and non-enzymatic antioxidant systems, that regulate the balance between antioxidants and reactive oxygen species (ROS) [22]. In this research, we reported phenolic composition and the antioxidant properties of *S. prostrata*.

Experimental

Chemicals

Reagent we used in antioxidant and HPLC methods were obtained from Sigma-Aldrich and Merck.

Identification and collection of the plant material

S. prostrata plant were collected from rocky slopes of Haserek mountain at 1800-1850 m altitude from Bingol, Turkey by Dr. Ömer Kılıç (herbarium number: 4764). according to the “The Flora of Turkey and East Aegean Islands, Volume 5” the taxonomic description was made [23].

Plant extraction

Extraction and purification of active plant ingredients materials demand a proper process [24]. *S. prostrata* plant were extracted considering our previous study[25]. For this purpose, 10 gram air-dried powdered-plant leaves were mixed with 100 mL ethanol and distilled water (1/10:w/v) homogenized with a magnetic stirrer (about 24 hours),

and filtered using filter papers. Using lyophilizer (Labconco, Freezone 1L) the frozen water solvent was lyophilized at -50 °C and 5 mm Hg. Using a rotary evaporator (Heidolph 94200, Bioblock Scientific) the ethanol solvent was evaporated. All of the samples were stored at -30 °C.

Antioxidant activity

FRAP assay

By the reduction of Fe^{3+} to Fe^{2+}, the reducing antioxidant effect of *S. prostrata* was determined as described previously [26]. Potassium ferricyanide (1%), phosphate buffer (0.2 M, pH 6.6), and $FeCl_3$ (0.1%) solutions were mixed. 10-30 μg/mL concentrations of extracts of the ethanol and water were added. Acidified with trichloroacetic acid (10%). Incubation was done at 50 ^oC for 20 min. The absorbance was measured at 700 nm.

CUPRAC assay

Cupric ions (Cu^{2+}) reducing ability of S. prostrata plant extract was carried out considering a well-known previous study. [27]. Acetate buffer (1.0 M), ethanolic neocuproine (7.5 mM), and $CuCl_2$ solution (10 mM) were mixed. 10-30 μg/mL of concentrations of the ethanol and water were added to mixture completed with with distilled water (2 ml). Then, the test tubes Incubation was done for 30 min at room condition. Absorbance was calculated at 450 nm.

DPPH assay

$DPPH^{\bullet}$ scavenging effect of *S. prostrata* was detected considering previously described work [28]. For this, 0.5 mL, 0.1 mM of DPPH solution and 1.5 mL of sample ethanol and water solution (10-30 μg/mL) mixed. Mixture left in dark for 30 min. Then absorbance was recorded at 517 nm.

HPLC assay

The phenolic composition of *S. prostrata* plant was analyzed by an advanced HPLC technique. Initially, plant extract was prepared to run on HPLC instrument. In a flask, 10 mg of S. prostrata extract was dissolved adding 1 mL of acetonitrile-water (v/v, 50:50) solution and homogenized 3 min with vortex. To separate and quantify the phenolic composition of the S. prostrata plant sample HPLC instrument was used. Each standard compound was prepared at a 10 mg/mL concentration and used for the standard graphs. To prepare stock solutions, acetonitrile and acetic acid (1%) were

mixed (1:9), then methanol was added to the mixture (1:1). For gradient elution, 1% acetic acid was used as solvent A and 100 % acetonitrile as solvent B [29].

Results and Discussion

Antioxidant activity

There are many different *in vitro* methods in the literature for the determination of antioxidant potentials of plants. These methods are commonly used to calculate food and plant antioxidant properties [30]. In the present study, we used DPPH assay to determine the radical scavenging activities of the extracts. By donating hydrogen, antioxidant substances reduce radicals and antioxidant scavenging abilities can be measure using spectrophotometric methods [31]. The radical scavenging level of samples demonstrates their antioxidant potential.

DPPH radical scavenging capacities of *S. prostrata* extracts were compared to the standard antioxidants. According to the DPPH method, the water extract of *S. prostrata* did not show significant scavenging activity on DPPH free radicals. However, the ethanol extract of *S. prostrata* showed considerable free radicals scavenging activity when compared to the standards used. It was observed in Figure 1, the free radical scavenging properties of the plant extracts and standards used increased with their concentration amounts. DPPH scavenging percentages of extracts and standards at 30 µg/mL concentration ordered as: water extract (2.2±0.2) < ethanol extract (26.3±4.0 %) < BHT (48.9±4.9 %) < BHA (85.2±5.9 %) < ascorbic acid (88.9±5.7 %). Also, the IC_{50} values of extracts and standards were found as; 108.6±9.7 µg/mL for water extract 36.8±14.6 µg/mL for ethanol extract, 11.5±5.2 µg/mL for BHA, 19.9±8.3 µg/mL for BHT, and 11.1±5.2 µg/mL for ascorbic acid.

The reducing power antioxidant activities of *S. prostrata* and standards were evaluated by both FRAP and CUPRAC reducing methods.

FRAP assay is a prevalent way to evaluate the reducing antioxidant ability. The antioxidant substance can change ferric (Fe^{3+}) ions into ferrous (Fe^{2+}). As seen in Figure 1, the reducing ability of the extracts was lower when compared with the standard reducing levels. The ethanol extract showed higher activity than the water extract. At certain concentrations, the reducing antioxidant capacities decreased as BHA > ascorbic acid > BHT > *S. prostrata* ethanol > *S. prostrata* water.

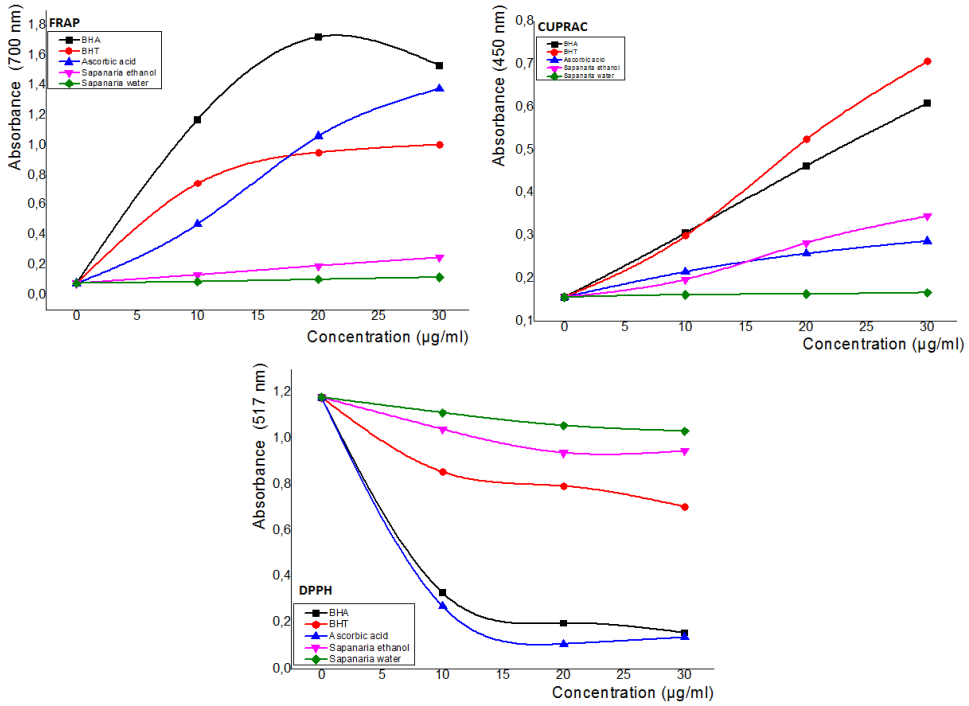

Fig 1 Antioxidant activity of *S. prostrata* and standards by using FRAP, CUPRAC, and DPPH methods

Cupric reducing the potential of *S. prostrata* was calculated using BHA, BHT, and ascorbic acid as standard. In the CUPRAC method, the ethanol extract of *S. prostrata* and standards showed remarkable reducing capacities. Ethanol extract antioxidant activity was higher than ascorbic acid at the 30 µg/mL concentration. However, the water extract showed very low activity. Reducing capacities decreased in the order of BHT > BHA > *S. prostrata* ethanol > ascorbic acid > *S. prostrata* water.

Phytochemical compounds

Polyphenolic compounds of plants have antioxidant and radical scavenging activity properties and are determined by the polymerization degree of the molecule and the number of active functional groups [32].

HPLC technique was used for the identification of the main organic compounds of *S. prostrata* by using different standard phenolic compounds. The results clearly showed the poor amounts of both total phenolic and total flavonoid contents of *S. prostrata.* According to the HPLC experiments, rutin (36.7 µg/g extract) and hesperidin (32.3 µg/g extract) were characterized as two major compounds in *S. prostrata*. Also, the low amounts of hyperoside, malic acid, and quinic acid were identified quantitatively.

The results demonstrated a high amount of rutin in the *S. prostrata* plant extract. It is clear that rutin extensively exists in fruits and vegetables [33, 34]. Due to its antioxidant, anti-inflammatory, and cytoprotective properties rutin is used in the treatment of many diseases [35]. Also, the potent DPPH scavenging ability and lipid peroxidation inhibition properties were reported for rutin as well [33]. As an antioxidant character, rutin could repair the damage of sodium perfluorononyloxy-benzenesulfonate to cell structures, and reduce the death rates of the bacteria under sodium perfluorononyloxy-benzenesulfonate exposure [36].

Conclusions

In this paper, we investigated and reported e phenolic content and antioxidant potential of *S. prostrata*. This work is a pioneer detailed study in describing and evaluating of chemical properties of *S. prostrata*. The results highlighted the potential role of *S. prostrata* to prevent the oxidation process. Therefore, further pharmacological investigations and *in vivo* tests are necessary to characterize and isolate plant active compounds.

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Research article

Cengiz, U., et al., Optimization of some parametric values of MTT for the determination of human melanoma (SK-Mel-30) cell viability. International Journal of Life Sciences and Biotechnology, 2022. 5(1): p. 9-20. DOI: 10.38001/ijlsb.991615

Optimization of some parametric values of MTT for the determination of human melanoma (SK-Mel-30) cell viability

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ABSTRACT

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazoliumbromide (MTT) assay is a widely used assessment method for the determination of anticancerogenic effects of active compounds including plant secondary metabolites. Recently, some important plant active ingredients have been widely investigated for anticancerogenic properties on melanoma cancer lines which are the most lethal type of skin cancer. Although some methods including DNA assay, ^{3}H-thymidine incorporation and flow cytometry have been recommended for counting cells in the culture, MTT is one of the most frequent method and therefore, MTT assay needs to be optimized for melanoma cell lines. In this study, the MTT analytical procedure for determination of cell viability of human melanoma cell line (SK-Mel-30) was divided into nine steps and various parameters in each step (reagent amount, incubation time, centrifugation, solvent type, waiting time before spectrophotometric analysis and spectrophotometric parameters) were optimized. Optimum amount of MTT reagent and incubation time after MTT addition were determined as 10 µL and 4 h for 96 well plate, respectively. Various solvents were evaluated for solubility effectiveness of the formed crystals and DMSO was found to be the best solvent to dissolve the crystals. Waiting time before spectrophotometric reading and Uv-vis spectrums were also evaluated. At the end of the study a flowchart, presented the best analytical conditions, was constructed. Obtained findings can be used for the determination of anticancerogenic properties of plant ingredients.

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Introduction

Malignant melanoma is the most widespread aggressive skin cancer type all over the world. Melanoma reports for 3% of all skin cancers but 66% of skin cancer - related deaths. The incidence of melanoma has accelerated by 340 percent since the 1950s; however, the mortality rate from melanoma has increased by only 150 percent. The majority of this is due to melanomas being diagnosed at an earlier phase when they may be more treatable. Melanoma is now the fifth most frequently diagnosed cancer in the United States. Over 70,000 invasive melanomas were diagnosed in the United States last year, with an added 53,000 in situ [1]. One in every 50 people will develop invasive melanoma in their lifetime. If in situ disease is considered, the lifetime risk is one in 34.

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Melanoma took the lives of 8790 Americans last year (approximately one dying every hour) [2]. According to the duration of exposure to sunlight Australia and New Zealand currently have the highest rates in the world (60 people in every 100,000 are diagnosed per year), with the USA (30 per 100,000 per year) and Scandinavia (20 per 100,000 per year) following. Approximately 104,350 new cases of melanoma were determined in the US and 11,650 persons are estimated to die from this cancer type in 2019 [3]. To control the disease and discover the novel drug therapies for the medication of the cancer needs the intensive research studies by using reliable and accurate methods [4]. Melanoma spreads rapidly to distant organs, making treatment tough. The rate of metastasis, its position, and the multitude of metastases all have a significant impact on patient survival, hence why it's critical to find a way to slow this process.[5, 6]

Nowadays, although many methods that can perform the counting cells such as fluorometric DNA assay, ^{3}H-thymidine incorporation and flow cytometry in the culture, one of the most widespread ways for determination of the viable cell number is the rapid colorimetric tetrazolium dye method commonly referred to as the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay [7]. Besides the cell viability, the test has been frequently used to determine cell proliferation and cytotoxicity because of its low cost, fast and simple test protocol [1, 7]. MTT is reduced by mitochondrial enzyme succinate dehydrogenase in mitochondria of metabolically active cells, and enzymatic reduction of the MTT results into the formation of water-insoluble purple colored formazan precipitate (Figure 1). Formed precipitate can be dissolved in a variety of organic solvents such as dimethyl sulfoxide (DMSO), isopropanol, sodium dodecyl sulfate (SDS) and absorbance of the resulting solution can be measured by a multi-well spectrophotometer [8]. In conclusion, measured absorbance values depend on mitochondrial activity gives an information about the number of metabolically active viable cells [7-9].

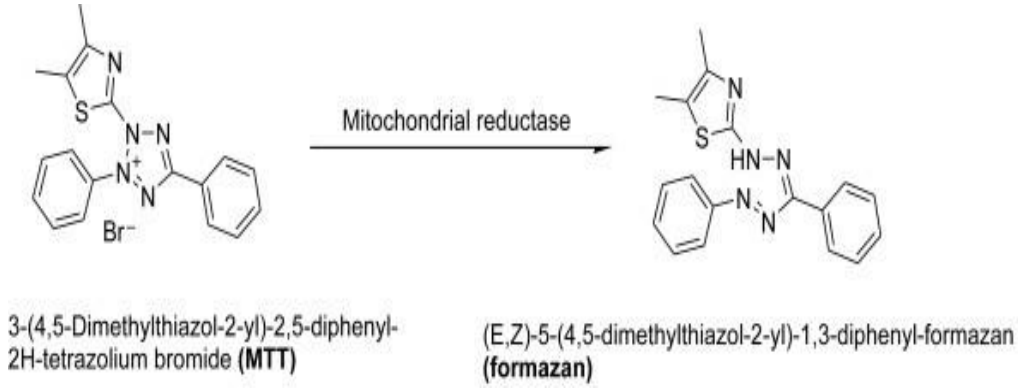

Fig 1 Enzymatic reduction of MTT to formazan [8].

Although, the method has been used in a number of laboratories with minor modifications, some parameters such as concentration and amount of MTT reagent, incubation time for production of formazan crystals, solvent types, centrifugation, cell numbers, waiting time before spectrophotometric analysis and parameters of the spectrophotometers such as wavelengths can affect to reliability of the MTT method [10]. The MTT test was shown to be sensitive to at least 1000 cells/well, whereas fewer cells can cause background high absorbance values [11]. It was also demonstrated that the absorbance signal increases in direct proportion to the incubation time, number of cells and amount of formazan crystals [12, 13]. One of the most important parameters of the MTT test is the solvents used to dissolve formazan crystals. Although the DMSO has been accepted as the most widely used solvent to dissolve formed formazan crystals, there are some other recommended alternative solvents such as isopropyl alcohol and acid isopropyl alcohol due to their lower background absorbance values than DMSO [7, 14]. Various organic solvents such as dioxane, cyclohexane, tetrahydrofuran, dimethylformamide were also used for the assay [15, 16]. In some previous reports, a centrifugation step was performed to precipitate formazan salts before solvent addition and medium removal to dissolve formazan salts [17, 18]. Although the MTT test is one of the effective and reliable method to measure cell viability, this analytical techniques used various kind of studies [19, 20] and it is necessary to optimize its accuracy and minimize the factors that affect MTT activity for specific cell lines. In the study, some critical factors that can affect the reliability and results of the MTT analysis were examined for determination of SK-Mel-30 cell counts in the culture.

Materials and Methods

Chemicals

MTT (Bio Vision Incorporated, California, United States) was dissolved in ultrapure water at the concentration of 10 mg/ml and filtered via 0.22 μm syringe filter (Gelman Sciences, Michigan, United States). The solvents (isopropanol and SDS) for the solubilization of MTT formazan crystals were obtained from Merck (Darmstadt, Germany). DMSO was purchased from ISOLAB (Wertheim, Germany).

Cell line

The human skin melanoma (SK-Mel-30) (ATCC, Virginia, United States) were plated in T75 tissue culture flasks and were cultured in High Glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, New York, United States) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Cromwell, United States), 1X Antibiotic/Antimycotic (50 units/mL penicillin and 50 mg/mL streptomycin) (Capricorn, Ebsdorfergrund, Germany), 1X MEM Non-Essential amino acids (Gibco, New York, United States).

Instrumentation

The cells were incubated for 24, 48 and 72 h at 37°C, 5% CO_2 in a culture incubator (Thermo Scientific, Ohio, United States). A centrifuge (Hettich, Tuttlingen, Germany) was used to pellet cells before removal of the medium. At the end of the MTT assay, absorbance of the resulting solutions was recorded at 570 and 690 nm using an ELISA plate reader (Thermo Fischer Scientific, Vantaa, Finland). The absorbance spectrum (450–700 nm) was performed using a Cary 60Uv-Vis Spectrophotometer (Agilent Technologies, Malaysia). Results were analyzed with the Soft max pro software (version 2.2.1). The ultrapure water was supplied by an ultrapure (18.2 MΩ cm at 25°C) purification system (Minipure, Ankara, Turkey).

MTT assay

A schematic diagram for MTT Assay was presented in Figure 2. According to the analytical procedure, SK-Mel-30 cells were plated in triplicate wells of a 96-well plate (**1**). The cells were then incubated at 37°C under an atmosphere 5% CO_2 and 95% humidity (**2**). After removal of cell media, each culture well was delicately washed with phosphate buffered saline (PBS) solution and fresh medium was added. In this step, various concentrations of active ingredient (i.e. 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024,

2048 µM) which are extracted from plant materials can be added into the well. The final concentration should be considered (**3**). Various amount of MTT solution (10 and 30µl/well) were added to the cells to find the optimum amount of solution (**4**). The cells were incubated for 2 and 4 hours at 37°C, %5 CO_2 (**5**). After incubation, 96-well plates were centrifuged or non-centrifuged, and medium (0%, 90% and 100%) was removed (**6**). Formed formazan crystals were dissolved in 5 different solvents (DMSO, %20 SDS, isopropanol, two different acid isopropanol concentration (0.01N HCl and 0.04 N HCl) (**7**). The plates were then waited for 3 different time intervals (0.5, 2 and 24h) in a dark place at room temperature (**8**). The absorbance was assessed at 570 and 690 nm by an ELISA microplate reader (**9**). All tests were repeated three times.

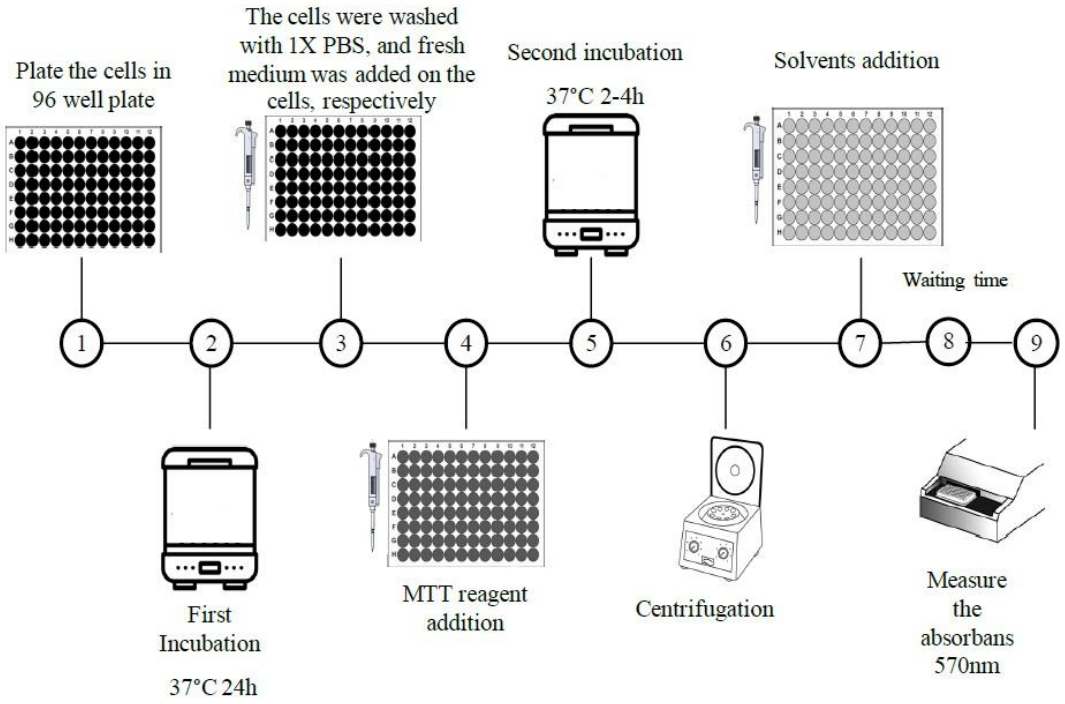

Fig 2 Workflow of MTT assay procedure.

Method validation

Method validation studies (limit of detection (LOD), repeatability and limit of quantification (LOQ)) were conducted according to the Eurachem guide [21]. The LOD was calculated at 3 times the standard deviation (SD), while the LOQ was calculated at 10 times the SD, which was obtained from the 10 independent analyses of samples with known counts of plated SK-Mel-30 cells (approximately 10,000 cells/well). Repeatability

was evaluated by assaying ten replicates of the individual MTT assay and was expressed as the relative standard deviation (RSD; %).

Statistical study

The data were expressed as means ± SD of at least 3 independent analyses. Significant differences were determined by one-way and/or two-way ANOVA followed by Tukey's multiple comparisons test employed by Minitab 17 statistical analysis program (Software, La Jolla, California). P value < 0.05 was considered statistically significant.

Results and Discussion

Preparation of the cells for MTT assay (Step 1-3)

The cells were plated at the concentration of $1x10^4$ number/well in 96-well plates (Step 1) and the cells were incubated at 37°C for 24h (Step 2). The number of the cell counts were in agreement with those reported by [22]. The medium in the wells were replaced with 1X PBS 24h later (Step 3). After the cells were grown in the cells, various concentration of plant active ingredients extracted and/or purified from the plants such as phenolic acids can be added to the cells. In this case, the final concentrations of active ingredients and living cell counts should be taken into consideration.

Throughout the first three steps of experimental workflow, schematized in Figure 2, the tested parameters were not changed. However, some parameters for the next steps were varied to find the most suitable parametric values.

Optimization of the other parameters (Step 4-9)

Effects of the other parameters (Step 4-9, illustrated in Fig 2) on the absorbance values for the MTT assay were evaluated. After the cells plating, the first incubating and washing procedure, various amounts of MTT solution was added to the cells in step 4. Two different volumes of MTT reagent (10 and 30 µL of 10 mg/mL) were selected in this experiment according to the various manufacturer protocols. The results showed that a significant increment in absorbance values were found related to the addition of MTT reagents. In the experiment, 10 µl of MTT solution was found to be a better concentration than 30 µl for production of formazan crystals as shown in Figure 3. The result were consistent with those reported previously [11, 23].

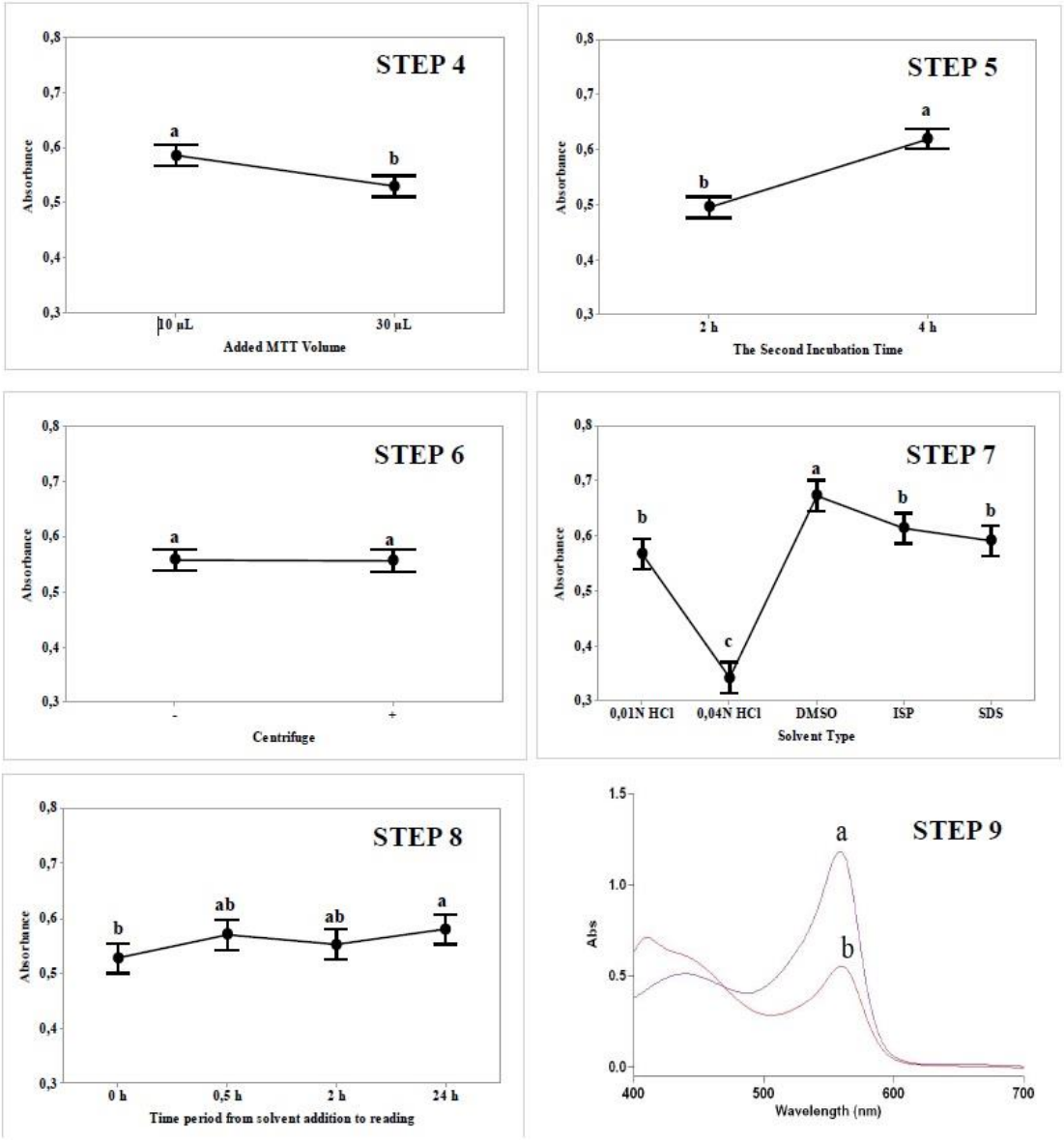

Fig 3 Affecting parameters of MTT assay for the determination of cell viability

After adding the MTT solution, the cells were incubated for two different time intervals as 2 and 4 hours for optimization of the second incubation time (Step 5). According to the results, the cells incubated for 4 hours showed a greater absorbance value than that of 2 hours (Figure 3).

A centrifugation procedure (500 x g for 5 min) was applied for the fast sedimentation of formed formazan crystals after the second incubation of the cells in the well plate (Step 6). To compare the sedimentation effects on the method performance, two different applications (centrifugation and without centrifugation) were performed. According to the results, there was no statistical difference among the absorbance values between with

and without centrifugation application (Figure 3, Step 6). Although centrifugation was recommended in many studies [17, 18], we could not achieved statistically significant results for centrifugation.

Small amount of formazan crystals, related to the viable cell counts, were observed at the bottom of the wells. The solubility of the crystals can be affected by medium fraction found in the upper phase of the wells when adding the solvent at the next step. Therefore, different amounts of medium fraction (0%, 90% and 100%) after the centrifugation procedure were removed from the wells and absorbance values of the crystals was evaluated. According to the results, the highest absorbance values were obtained in the experiment of without removing of the medium (0%), whereas the lowest value was found in 100% removing medium. It can be attributed to the additional absorbance values of the medium may increase the total absorbance value. To find the exact value of absorbance that can be result from the formazan crystals the medium content in the upper phase must be removed from the well. This procedure can increase the dissolving of formazan crystals when adding the solvent at the next step.

Various solvent types (DMSO, %20 SDS, isopropanol, two different concentration of acid isopropanol (0.01N HCl and 0.04 N HCl)) were applied to dissolve the formed formazan crystals (Step 7). The results showed that the lowest absorbance value was obtained for isopropanol with 0.04N HCl application. As shown in Figure 3 Step 7, there was no statistically significant difference among the SDS, ISP and ISP with 0.01N HCl ($P<0.05$). The highest absorbance value was achieved for the application of DMSO. It was reported that the DMSO [24, 25] and acid-isopropanol (0.04 N HCl in isopropanol) [7, 26] were widely used for the dissolving solvents of formed formazan crystals.

DMSO is one of the important organosulfur compounds with the formula of $(CH_3)_2SO$. This compound has a polar character that dissolves both nonpolar and polar compounds and is miscible in a wide variety of organic solvents as well as water. DMSO has a relatively high boiling point. It is also a reliable compound for toxicological aspects and it has a median lethal dose higher than ethanol (DMSO: LD_{50}, oral, rat, 14,500 mg/kg; [27], ethanol: LD_{50}, oral, rat, 7,060 mg/kg [28]).

Absorbance values in wells were measured at various time intervals after the addition of DMSO to find the effects of waiting periods (Step 8). The lowest absorbance value was observed following addition of medium within 0.5 h. There was no statistically difference

between the 0.5 and 2 h for the absorbance values, but that the values increased for 24 h (Figure 3, Step 8). After the MTT formazan dissolved in DMSO, the optical density remained the same for several hours, but the resolution increased after 24 hours. In conclusion, prepared solutions should be read within 2 hours. Obtained result agreed with the study of [29].

After the waiting period obtained absorbance was read by a spectrophotometer at the wavelength of 570 nm. Although the formed formazan crystals in DMSO gives some absorbance values in spectrophotometers, used medium and DMEM result in additional absorbance values as shown in Figure 4. According to the figure, DMSO has no absorbance value in the tested wavelength range (a) and formazan crystals solved in DMSO has an absorbance value depending on the viable cell number. Therefore, the medium fractions which are found in upper phase after the centrifugation step before the addition of solvents should be carefully removed to increase the absorbance value for the formazan crystals. In addition, absorbance values of DMEM (b), MTT (c) and FBS + DMEM (d) were found to be higher than the DMSO absorbance. The results show that additional absorbance values can be observed when these compounds are used in experiments. Therefore, these values should be considered as control samples.

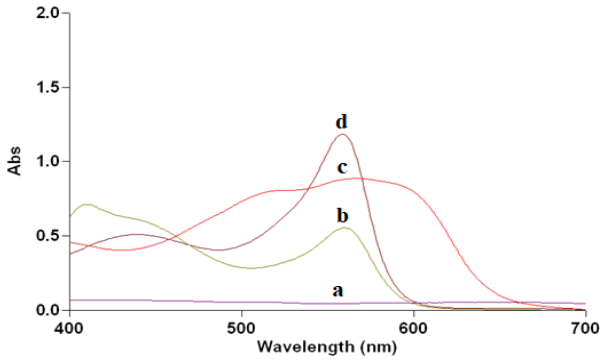

Fig 4 Absorbance values of DMSO (a), DMEM (b), MTT (c) and FBS + DMEM (d)

Method validation

Repeatability of the method was found in the range between 4.939%. LOD and LOQ values were found to be 495 and 1651 cells/well. As a result, the optimized method can give reliable results if the cell number is higher than 1651 cells/well.

Conclusion

The metabolic activities among different cell lines have been reported a great variability and therefore MTT assay needs to be characterized and optimized for melanoma cell lines. In the assay, metabolically active cells can convert the MTT dye (yellow water-soluble tetrazolium salt) in water to insoluble dark formazan crystals by reductive cleavage and the conversion can be affected by many factors during the analytical procedure. MTT assay has great potential as a fast method for determining cell viability of cell lines. In this study, a tetrazolium salt (MTT) based colorimetric assay was optimized and validated. Constructed flowchart based on the analytical results was presented in Table 1. Optimized MTT assay can be reliably applied to determine cell viability of SK-Mel-30.

Table 1 Constructed flowchart based on the analytical results

Step	Actions	Optimum parameter
1	Seeding the cells	10000 cells/well
2	First incubation	37°C 24h
3	Washing the wells	The medium is replaced with 1X PBS after 24h
4	MTT reagent addition	10 µl from 10 mg/ml
5	Second incubation	37°C 4h
6	Centrifugation	No difference
7	Solvent addition	DMSO
8	Waiting time for reading	0.5-2 hours
9	Wavelength	570 nm

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Research article

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3D Structural Prediction of Catechin Specific Aptamer

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ABSTRACT

Catechin has been reported to possess many advantageous for practical application due to its distinctive antioxidant and anti-inflammatory activities. This paper reports the *in-silico* characterization of single stranded-DNA (ssDNA) aptamers, specific for catechin. 28 primary sequences from DNA-aptamers library screened via systemic evolution of ligands by exponential enrichment (SELEX) from previous research were predicted and constructed into 3D structural conformation using several bioinformatics tools. Blind docking simulation was performed to all 28 aptamer candidates and resulted in 4 noticeable aptamers with highest binding energy arrangement from Aptamer 24, 18, 9 and 27. Influence of external factors towards catechin specific aptamers also was taken in consideration. It was predicted that these aptamers were the most potential aptamer for catechin recognition tool at laboratory scale based on the docking result. However, further *in vitro* experimental study in laboratory needs to be done as validation.

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Introduction

The catechin is one of naturally found polyphenol which is known as major component of a few medicinal [1] and various plants especially green tea and fruit-based products [2]. The most common isomer known is the (+) catechin. Due to its beneficial antioxidant characteristic, it has a wide application in health and medicine. The antioxidant and antimicrobial properties in catechin are reflected to the presence of a gallate moiety at third position of C ring which increases radical scavenging activities [3]. Besides, catechin is known due to its advantages as having hepatoprotective effect, antihypertensive, antiinflammatory, antiallergic, antithrombogenic, and hypolipidemic properties [4]. Thus, the recognition of this cellular-level compound needs to be enhanced for the sake of its advantages.

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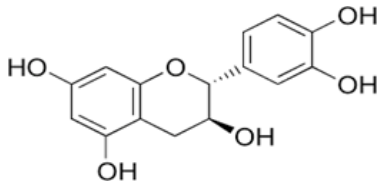


Fig 1 The 2D structure of cathechin used in this study

Therefore, a technology called aptamers is used as it can bind to their target compound through specific 3D-conformation [5]. Aptamers are short oligonucleotides with highly structured molecules which are ten times smaller than antibody, easily select, simpler and more robust than antibody-based probes. It is originated from *in vitro* experiments termed as systematic evolution of ligands by exponential enrichment (SELEX). This system is started from the libraries of random sequence and the optimization of nucleic acids to increase the binding affinity to the expected ligands. Aptamers are predominantly without structured in a solution or liquid form. Once associating with ligands, aptamers start to fold into molecular architectures and the ligand becomes nucleic acid intrinsic component [6].

Initially, the prediction of 2D structure involves the input of DNA sequences that were resulted from post-SELEX procedure or PDB online database searching with a focus to the occurrence of hairpin-like structure of single-stranded DNA (ssDNA). The process begins with prediction of ssDNA's secondary structures using Mfold web server [7]. The most thermodynamically stable structures are select from Mfold and all possible secondary structures resulted are approximately based on Watson-Crick base pairing. Construction of 3-D structure of ssDNA aptamers involved multiple software such as RNA composer [8]; Discovery Studio Visualizer and UCSF Chimera [9]. In purpose to remove deficiencies in geometric and maximize the structural parameter, the 3D RNA models then need to be refined. The ssRNA is modify into ssDNA 3D structures by detecting every uracil residue to substitute with methyl and the ribose sugar backbone replace is with deoxyribose. Final stage of the procedure involves the refinement of ssDNA 3D structures, validation of structure [10] and the predicted structure are ready to be analyzed.

Material and Methods

Structural Prediction of DNA Aptamers

The sequence of aptamers gained from previous research were converted into two-dimensional structure [11]. This process was conducted using the Mfold web server [7]. The temperature, monovalent and divalent ions concentration were adjusted to 25°C, 2 mM and 100 mM, respectively. The resulted structures were saved in portable network graphic format (png) and

Vienna file. The structures produced were validated by using Mathew Lab web server. The output from Mfold were used as an input for further analysis of 3-D structure prediction using RNA Composer. The modification method in Discovery Studio Visualizer DSV) involved several steps which include the conversion of bases (uracil into thymine) and sugars (ribose into deoxyribose). Finally, the structures were optimized at 30,000 steps of steepest descent energy minimization method and followed by 10 steps of conjugate gradient using UCSF Chimera 1.13 [9].

Docking analysis using AutoDock Vina

Digital ligands files in pdb format of catechin (compound ID: 73160) was downloaded from the PubChem database. The receptors (ssDNA aptamers) that already optimized previously was used for docking. Docking was performed in AutoDock Vina [12], which can predict interactions between small molecules and nucleic acids.

Assessment of the environmental effects of Catechin-specific aptamer

Resulted from docking process, four aptamers with the highest score of binding energy were selected as catechin-specific aptamers. Then, all of these aptamers were constructed again from their sequence by setting the parameter of temperature and other factor such as Mg^{2+} and Na^{+} using default setting of Mfold web server. The temperature, Mg^{2+} and Na^{+} default parameters were 37 ℃, 0 and 1 M respectively. The output of Mfold server of these for aptamers were then converted into 3D-DNA and were analyzed for docking analysis.

Results and Discussion

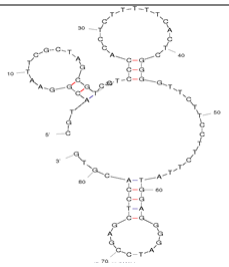
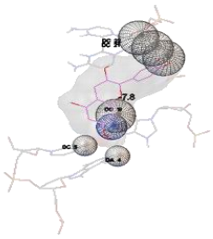
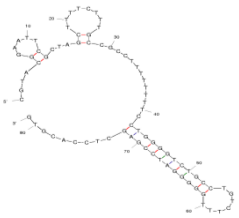
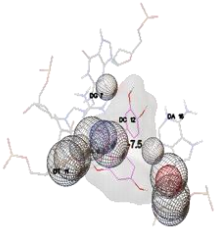
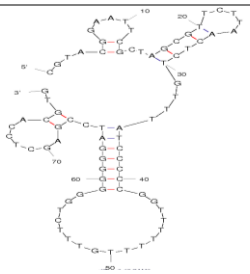
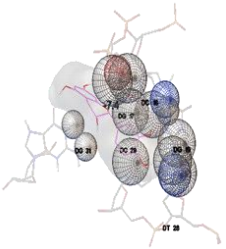
Three-Dimensional Structure and Docking of DNA Aptamers against catechin

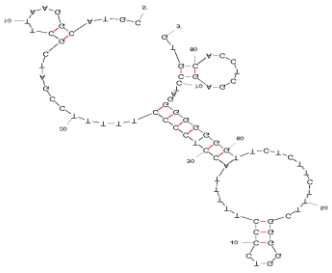
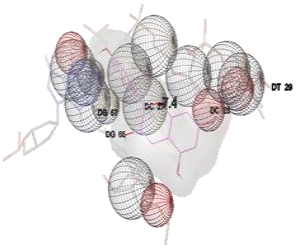
The resulted structures of aptamers were all folded to its conformation with the adjustment of the temperature at 25℃, 2 mM Mg^{2+} and 100 mM Na^{+} concentration to the most favorable condition resulting from previous *in vitro* SELEX procedure. Divalent ions such as Mg^{2+} has crucial roles in facilitate the folding of ssDNA constructed as it affects the interaction of DNA with its ligands or other protein. Furthermore, it is an essential cofactor in enzymatic system in DNA processing [13] with additional stabilizing effect. İn contrast, Na^{+} ion involves in controlling the electric field of DNA surface which affects the charge neutralization and the ion binding of the DNA structures gained [14]. The temperature were adjusted as its affect the flexibility and compactness of the DNA fold by interfering the bending of chromatin [15]. Among 28 aptamer candidates, top 4 highest binding energy with hydrogen bond location were identified and listed in Table 1.

Evaluation of the binding activity of aptamers to catechin via docking

Autodock Vina output provides the information for the H-bonds position, binding energy of the aptamer-catechin complex and nucleotides location of the ligand attachment as shown in Table 1. Each of the aptamers recorded different strength of binding affinity and binding location. These happened due to the contribution of a lots of factors such as the temperature, Mg^{2+} and Na^{+} ion concentration, Gibbs free energy and the structural conformation of the DNA aptamers. Temperature is not necessarily the only factor in aptamer performance, but it is importance in selecting the right temperature for downstream applications of aptamers. The diagnostic aptamers at room temperature should always be fixed in a range of 20–25 ˚C and the therapeutic aptamers should be static at body temperature which is 37˚C to ensure the best result for the final application of the DNA aptamers [16].

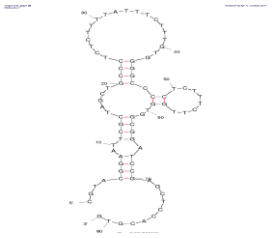
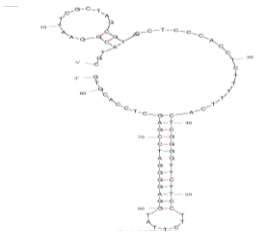
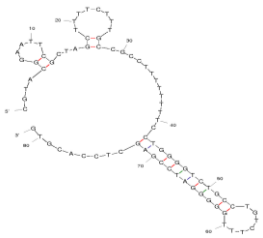
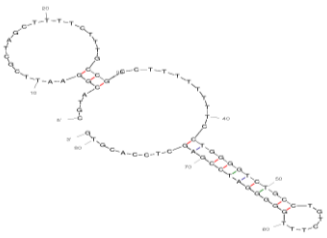
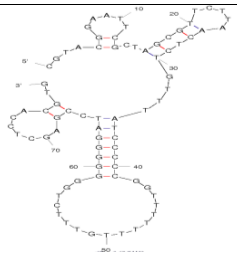
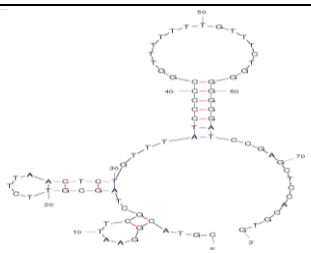
Table 1 Structural prediction and docking energy of ssDNA aptamers against catechin

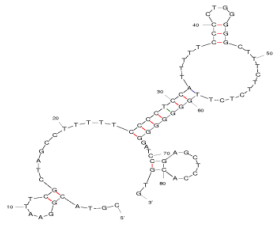
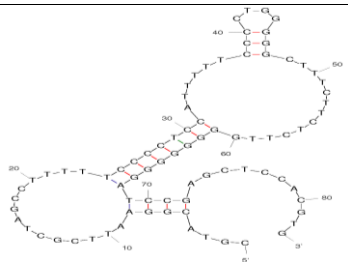
Aptamer	2D structure of possible aptamer candidates	Highest binding energy, (kcal/mol)	Site of binding	Hydrogen bond location
24		-7.8		DC5:H42
18		-7.5		Absent
9		-7.4		Absent

27		-7.4		Absent

Other than that, the concentration of Mg^{2+} and Na^{+} ions play an important part in the strength of binding of DNA aptamer. The Mg^{2+} ion is vital in facilitating the folding of aptamers and interaction of aptamers with its ligand, catechin [14]. While the Na^{+} responsible in controlling the electric charge of the DNA aptamer surface which may cause the shielding effects of negative charges of aptamer and could result in a decline of the binding affinity [17].

Table 2 Environmental assessment on catechin specific aptamer

APTAMER	**2D STRUCTURES**			
	Adjusted parameter	**Binding energy (kcal/mol)**	**Default parameter**	**Binding energy (kcal/mol)**
29		-7.8		-6.9
18		-7.5		-6.9
9		-7.4		-7.5

27		-7.4		-6.8

In addition, the structural conformation of the aptamer affect the affinity of binding and their functional contacts to catechin due to its hydrophilic polyanionic backbone and lack or exhibit weak hydrophobic interactions [18]. The binding affinity of aptamers is thermodynamically related to the Gibbs free energy (ΔG). All the structural conformation of aptamer chosen from the generated structure by Mfold web server was the one with the lowest ΔG since the more negative value is the most stable structure. Thus, when aptamer bind to ligand with a perfect fit, binding affinity could be maximally enhanced by entropic optimization. In contrast, small structural mismatches may lead to very weak binding affinity due to a negative impact on enthalpy [19]. The obtained docking results confirmed the capability of DNA aptamers possess effective molecular interaction with catechin.

Environmental assessment on Catechin specific aptamers

The assessment of the catechin specific aptamers has been carried out to observe the effect of environmental changes towards their structural conformation and activity of the DNA aptamer with its ligand. From the result observed in Table 2, the aptamers with the changed parameter structural are differ compare to the structural of the original aptamers. The binding energy of original parameter were declined as compared to changed parameters. İt might be due to the result from the folding of the structure as the aptamers experienced differentiation in the size and number of loops.

Binding affinity of hairpin DNA is mainly determined by the size of loop and the integrity of stem. Folding of DNA hairpin occurs through complicated reaction mechanism that involves both long-lived and short-lived reaction intermediates [20]. Although the hairpin loop possessed minor influence in specific site interactions on the stem, but it significantly affects the non-specific binding. In general, larger loops decreases the stability of the hairpin while smaller hairpin is more stable [21]. However, the study on this has received little attention which resulted in fewer findings in proving the effect of the loops and stem in the structural conformation of DNA aptamers towards the binding affinity.

Conclusion

The applications of DNA aptamers as biorecognition tools are expanded to its ability to detect selected flavonoids. Our study shows that the intermolecular interaction between specific DNA aptamer against selected flavonoids namely catechin, luteolin and kaempferol can provide a basic understanding on binding orientation in the complex. This was achieved by performing *in silico* approach in 3-D structure prediction and docking analysis.

Abbreviations

DNA: deoxyribose nucleic acid; RNA: ribonucleic acid; 3-D: three dimensional; PDB: Protein Data Bank; Mg^{2+}: magnesium; Na^{+}:sodium

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Research article

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Yoghurt production using pinecone and investigation of the effect of the produced yoghurt on ECV304 cell line

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ABSTRACT

The goal of this study was to ferment yoghurt from milk using lactic acid bacteria found in pinecones and to examine the cell viability and lipid peroxidation levels of the fermented yoghurt on Vascular Endothelial Cell Line ECV304. ECV304 was cultured in vitro. To determine cell viability, the various concentrations of yoghurt extract fermented with pinecone were given to cultured cells using the MTT Assay. At the same doses as the MTT Assay, the Lipid Peroxidation (LPO) Assay was employed to evaluate the malondialdehyde (MDA) levels of cells. Acridine orange/Ethidium Bromide staining technique was applied to detect apoptosis. Gas Chromatography-Mass Spectrometry was used to identify the volatiles in yoghurt fermented with the pinecone. All dosages of pinecone fermented yoghurt enhanced the cell viability of the human healthy vascular endothelial cell line ECV304 and decreased MDA levels, as validated by fluorescence microscopy pictures. The primary essential oils identified in yoghurt fermented with pinecone were hexadecanoic acid, eicosanoic acid, stearic acid, and 2-palmitoylglycerol. In this work, the effects of yoghurt fermented with pinecones on human healthy cell lines were examined for the first time. The study discovered that this yoghurt promotes the proliferation of healthy human cells while decreasing oxidative stress in these cells.

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Introduction

Yoghurt (yogurt) is well known as a highly preferred product among foods around the world [1]. This substance production has began centuries ago; therefor several hypothesises illustrates how the first synthesis of yoghurt took place through the course of history [2]. Milk was fermented in ancient times to prevent it from spoiling. Although no record of the earliest fermentation of milk exists, it is considered to have occurred in the Middle East prior to the Phoenicians' life. [3] The stages of yoghurt manufacture have been passed down from the old to the young. There is no doubt that yoghurt is a legacy, particularly in recent years, with the advancement of technology in the food business, the consistency, flavor, and density of yoghurt have acquired a specific standard. [3] Yoghurt

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production, in general, is a modification of the original compounds of the milk, application of pasteurization to the yoghurt mixture, fermentation by incubating at optimum temperature, cooling; adding flavor or fruits can be achieved as an additional step [4]. According to the Codex standard published by the FAO/ WHO (The Food and Agriculture Organization of the United Nations/ World Health Organization), it is obtained because of fermentation of milk thanks to some lactic acid bacteria [5].

Streptococcus thermophilus is a gram-positive bacterium. *Streptococcus thermophilus* is a fermentative facultative anaerobe. It does not move and does not form endospore. It has an optimum growth temperature range of 35-42 ℃. It is also classified as lactic acid bacteria. *Streptococcus thermophilus* is found in products of fermented milk and is often used in yoghurt production. The major bacterium used in yoghurt manufacturing is *Lactobacillus delbrueckii subsp. bulgaricus.* It can also be present in naturally fermented foods. Stamen Grigoro, a Bulgarian scientist, discovered *Lactobacillus delbrueckii subsp. bulgaricus* bacterium in 1905. *Lactobacillus delbrueckii subsp. bulgaricus* bacteria generate lactic acid by feeding on lactose. The optimal temperature range for *Lactobacillus delbrueckii subsp. bulgaricus*' growth is 43-46 °C [6]. *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* are yoghurt fermenting lactic acid bacteria. These two kinds of bacteria have a symbiotic connection. *Streptococcus thermophilus* multiplies fast at the start of fermentation, generating pyruvic acid, formic acid, and carbon dioxide [7]. *Lactobacillus delbrueckii subsp. bulgaricus*'s growth is aided by pyruvic acid, formic acid, and carbon dioxide. *Lactobacillus delbrueckii subsp. bulgaricus* hydrolyzes milk protein at the same time. *Streptococcus thermophilus* grows as a result of this occurrence. Bacterial cultures produce lactic acid from lactose, the primary carbohydrate in milk, during the fermentation process. Lowering the pH and protein levels in milk causes coagulation because milk has a solid-gel-like structure [8]. Milk components; it is broken down into carbonyl compounds, nonvolatile acids, and volatile acids [9]. Acetaldehyde, acetone, acetoin, diacetyl, and acetate are a few examples. These contribute to the distinctive flavor of yoghurt [10].

On plants, a pinecone is an organ with a reproductive system. Pinophyta contains pinecones (conifers). The female pinecone produces seeds, whereas the male pinecone generates pollen. It's often herbaceous [11]. Pinecone is utilized as a treatment technique in respiratory disorders such as sputum and asthma, according to the Materia Medica

Compendium book was written by Li Shizhen, [12] which provides herbal medical knowledge historically used in China. Furthermore, pinecone has been found to have a possible therapeutic impact in clinical treatment techniques for illnesses such as cough, malignancy, viral infection, neurasthenic, and intestinal inflammation [13]. Studies have observed that the polyphenols in pinecones are beneficial for human health [13-14-15]. According to Yi et al.'s (2017) research, pinecone possesses anticancer and antioxidant effects due to the different polyphenols found in it [15]. *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* were found in both yoghurt and pinecone in research conducted by Bostan et al. (2017) [16]. However, to the best of our knowledge, no research has been conducted on the fermentation of yoghurt using a pinecone. In this work, ECV304 was employed as a healthy cell line. ECV304 was transformed by itself derived from a Japanese human umbilical vein endothelial cells (HUVEC) culture. ECV304 cell line is a healthy cell line [17]. Here in; because the pinecone contains a polyphenol, if yoghurt can be fermented effectively, cell viability studies on ECV304 will be performed, and the impact of yoghurt on human healthy endothelium cell line will be examined.

Materials and Methods

Materials and chemicals

The following materials were used for the fermentation and extracting procedure; immature pinecones, daily cow's milk and 0.22 μm filter (ASIMO). Fetal Bovine Serum (FBS) (Gibco, Cat No. 10500) and Dulbecco's Modified Eagle Medium (DMEM) (Gibco, 11960044) were used for culturing the cell. MTT ((3- (4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide) was obtained from Invitrogen (Thermo Fisher Cat No: M6494). All of the other compounds utilized in the investigation for instance Phosphate Buffered Saline (PBS) (VWR, (pH: 7.4) Cat No: E504), Dimethyl sulfoxide (DMSO) (VWR, Cat No: 23500.322), Isopropanol (VWR, Cat No: 20842.323) and Acridine Orange Base (Sigma-Aldrich, 235474-5G) were of analytical quality and purchased from commercial sources.

Collection, transport, and storage of pinecones

Immature pinecones were collected on April 18, 2020, at 11:00 a.m., off the Gökköy Village (39°44'47.6"N 28°21'07.6"E) of Kepsut District in Balıkesir Province. They were placed in a plastic bag and sent to the PROMER (Istanbul Protein Research Development

and Innovation Application and Research Center) Laboratory at Üsküdar University. The pinecones submitted to the lab were kept at -20 °C.

Fermentation of pinecone yoghurt

3 liters of daily cow's milk were purchased at Ovit Yaylası Yöresel Süt Ürünleri Store in Ümraniye District, Istanbul Province (41 ° 01'23.6 "N 29 ° 06'26.4" E). As a consequence of the chat with the vendor, it was discovered that the daily milk purchased is not from a single cow, but rather is sold by blending the milk of several cows. Pinecones were dissolved in a 25 °C water bath (Nuve ST30) at -20 °C. 250 mL of milk was transferred to a sterile beaker and cooked for 1 hour and 20 minutes on a magnetic stirrer (Isotek). It was allowed to cool at room temperature until it reached 44 °C. 45 grams of pinecones, in pieces, were dropped into the milk. With a minor modification on the work of Bostan et al. (2017), It was incubated for around 7 hours at 47 °C by covering the top with aluminum foil. The fermented milk that was converted into yoghurt was kept at +4 °C until the next usage [16].

Preparation of pinecone yoghurt extraction

The precision scale (Radwag AS 220/C/2) was used to weigh 35 grams of pinecone yoghurt, which was then placed into a sterile falcon. It was centrifuged (Beckman Coulter Allegra X-30R) for 60 minutes at 3901g at +4 °C, and the operation was done twice. The supernatant was filtered twice through an 0.22 μm filter (ASIMO) before being kept at -20 °C until use [18].

Cell viability assay

Cells were counted, and medium containing 10% Fetal Bovine Serum was given to cells to achieve a cell density of 100.000 cells per mL. A 96 Well Plate (Nest) was seeded with 90 μL cells. The 96 Well Plate was incubated for 24 hours at 37 °C and 5% CO_2. After 24 hours, 10 μL of pinecone yoghurt doses were added, with doses customized to each well. According to Chen et al. (2007), the yoghurt doses were 0,31%, 0,63%, 1,25%, 2,5%, 5%, and 10% (vol/vol) [19]. The 96 Well Plate was incubated for 24 hours at 37 °C and 5% CO_2. Each well received 10 μL of MTT (Invitrogen, Cat No: M6494) solution with a stock concentration of 5 mg/mL prepared in sterile PBS (Phosphate Buffered Saline) (VWR, (pH: 7.4) Cat No: E504). It was incubated for 3 hours at 37 °C and 5% CO_2. After 3 hours, 80 μL was removed from the wells without contacting the cells, and 90 μL of 50% DMSO (VWR, Cat No: 23500.322) - 50% Isopropanol (VWR, Cat No:

20842.323) was added to each well. Aluminum foil was used to cover the surface of the 96 Well Plate. For 45 minutes, the 96 Well Plate was kept at room temperature. The 96 Well Plate was measured at 570 nm using a microplate spectrophotometer (Thermo Scientific Multiskan GO) after 45 minutes [20].

Evaluation of MDA levels by TBARS assay

Thiobarbituric acid reagent (TBARS) analysis was used to determine the cells' malondialdehyde (MDA) levels. Cells were collected with the aid of lysis buffer after being cultured for 48 hours with the specified doses, after washing with PBS. For 30 minutes, the collected cells were incubated at +4 °C. The mixture of 50% TBARS Acid Reagent and 50% cell was then kept at room temperature for 15 minutes. The samples were then centrifuged. The MDA Standard was made by combining TBARS Standard with TBARS acid Reagent (1: 2) and leaving it at room temperature for 30 minutes at 180 rpm. By serial dilution between 167 µM and 0 µM concentrations, 5 MDA Standards were produced. Both samples and MDA Standards were applied as 150 µL to the 96 Well Plate. After that, 75 µL of TBA Reagent were pipetted into each well gently. A microplate reader (Thermo Scientific, USA) was used to obtain two readings at 532 nm at 2.5hour intervals [20].

Fluorescence microscopy

The morphological analysis was observed using dual AO/EB staining. Acridine Orange Base (Sigma-Aldrich, 235474-5G) was dissolved in distilled water to make a solution with a concentration of 1 mg/mL. This prepared solution was combined with 900 µL of sterile PBS (VWR, (pH: 7.4) Cat No: E504). 900 µL of sterile PBS was mixed with 10 µL of Ethidium Bromide (EtBr). The acridine orange solution was mixed with sterile PBS in a 1:1 ratio, and Ethidium Bromide was mixed with sterile PBS. All of the cell's medium was discarded. The cells that had adhered to the flask were washed twice in 1 mL of sterile PBS. 200 µL of AO/EB solution prepared at a 1: 1 ratio was added to the cells in the flask, and the solution was distributed over the whole flask surface. For 20 minutes, the flask was incubated at 37 °C and 5% CO_2. On the coverslip, 25 µL of AO/EB stained cells were placed. A fluorescent microscope (SOIF Optical/MF52) with a 20x objective and a 10x ocular was used to observe cells [21].

GC-MS analysis

A 100 µL yoghurt extract sample was placed in the test tube. After that, 900 µL MSTFA (N-Methyl-N-trimethylsilyl tri fluoroacetamide) was added, and the mixture was mixed for 1 minute using a vortex mixer before being maintained at 70 °C for 30 minutes. The mixture was filtered using a 0.22 µm filter. The filtrate was injected into the GC system in 5 µL increments.

GC-MS parameters

Essential oils were analyzed by using a Shimadzu GC-2010 plus gas chromatography (Shimadzu Scientific Instruments, Columbia, MA, USA), equipped with an Rtx®-5MS column (30 m × 0.25 mm ID, 0.10 µm film thickness) (Restek, USA). Helium was used as carrier gas (average flow rate, 1.50 mL/min). The oven temperature program was set as the following: keeping at 150 °C for 4 minutes, ramping at 50 °C min^{-1} up to 200 °C and keeping at 200 °C for 2 minutes, then ramping at 1 °C min^{-1} up to 250 °C and keeping at 250 °C for 18 minutes. This led to a total run time of 75 minutes. The temperatures of the transfer line and ion source were set at 250 and 200°C, respectively. Mass spectra were scanned from 40–400 m/z at a rate of 0.5 scans s^{-1} and the electron impact ionization energy was 70 eV. Data handling was supported by the software GC-MS solution, ver. 2.51 (Shimadzu). The compounds of extracts were identified using the National Institute Standard and Technology (NIST) library. The relative percentages of the compounds were calculated based on the GC peak areas.

Results

Fermentation of pinecone yoghurt

The pinecone-fermented yoghurt had the same consistency and color as commercially available yoghurts. Unlike commercial yoghurt, pinecone yoghurt contained a little quantity of pinecone taste and fragrance.

Cytotoxicity results

Figure 1 shows the cell viability findings of a pinecone fermented yoghurt ECV304 cell line at various doses. Cell viability gradually increased in the range of concentrations of 0,63% - 10 (vol/vol) except for the 0,31% (vol/vol) doses as compared to the control group, according to the findings of the MTT Assay performed on ECV304. The 0,31% (vol/vol) dose had a similar effect on cell viability as the 1,25% (vol/vol) dose. The dose

of pinecone yoghurt with a concentration of 10% (vol/vol) stimulated proliferation the greatest.

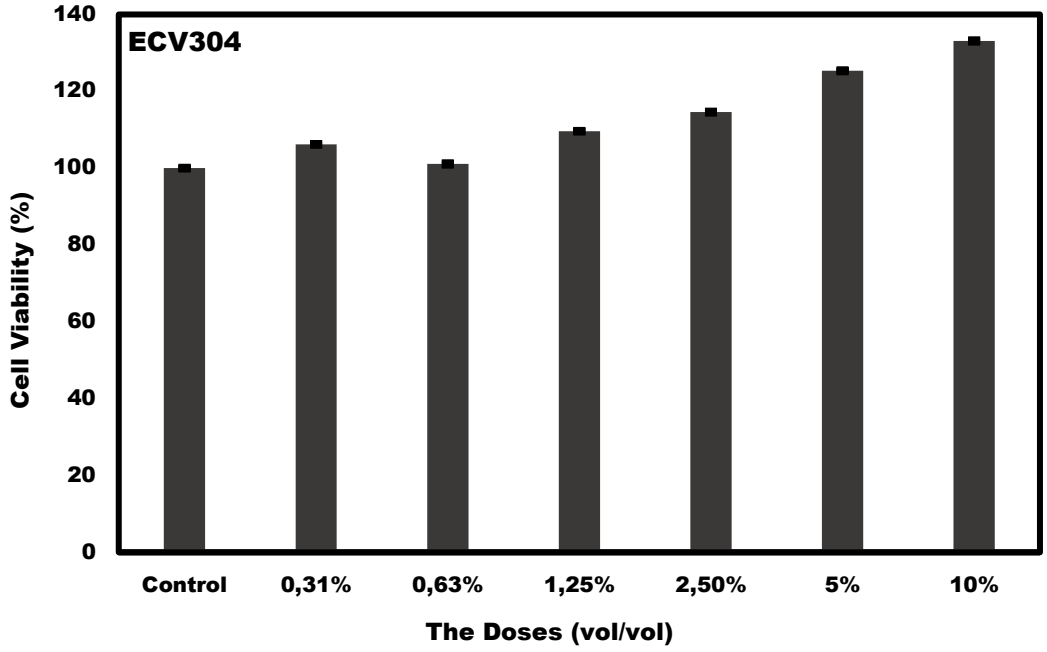

Fig 1 Cell viability results using MTT assay for ECV304 cell line (n= 6)

Lipid peroxidation levels

Figure 2 depicts the percent malondialdehyde (MDA) levels corresponding to lipid peroxidation levels affected by treatment with the ECV304 cell line of yoghurt fermented with various pinecone concentrations. When compared to the control group, all doses supplied decreased MDA levels. The dose with a concentration of 1,25% (vol/vol) lowered the MDA level of the ECV304 cell line the greatest. MDA levels reduced at a concentration of 0,31% (vol/vol) as compared to other doses.

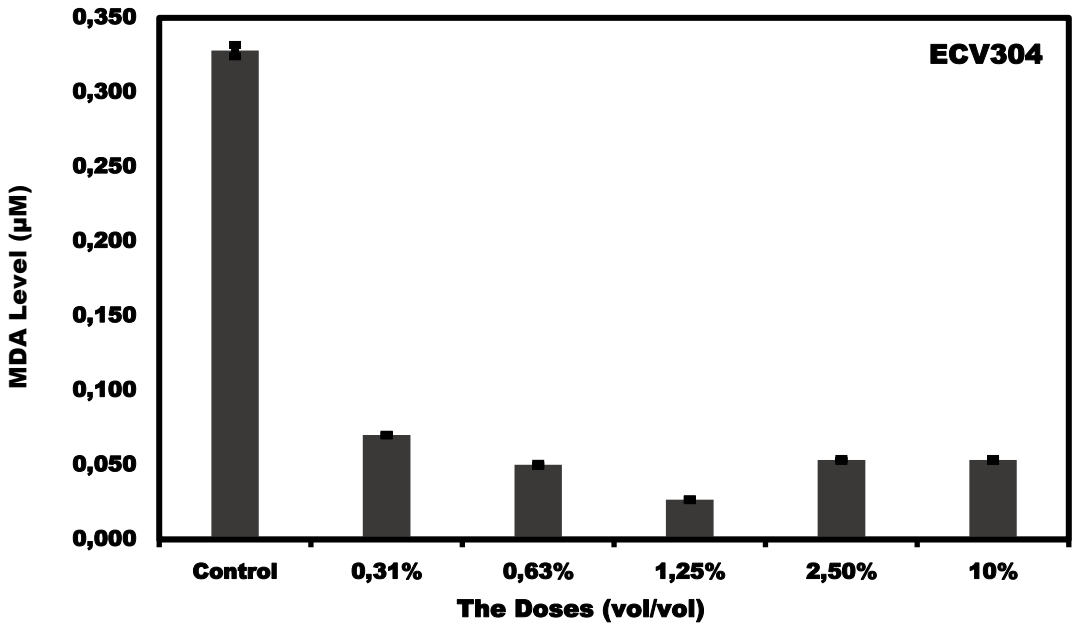

Fig 2 MDA levels affected by various concentrations of pinecone yoghurt in ECV304 cell line (n= 5)

Fluorescence microscopy

Fluorescence microscopy images of the ECV304 cell line after treatment with various doses of pinecone yoghurt are shown in Figure 3. Pinecone yoghurt was administered to the ECV304 cell line at several doses and no apoptosis or necrosis was detected. In comparison with the control group, there was no significant increase in cell proliferation at a dose of 0,31% (vol/vol). ECV304 cells, on the other hand, did not undergo apoptosis or necrosis when given this dose. Concentrations of 10%, 5%, and 2,50% (vol/vol) in particular were shown to contribute to cell division.

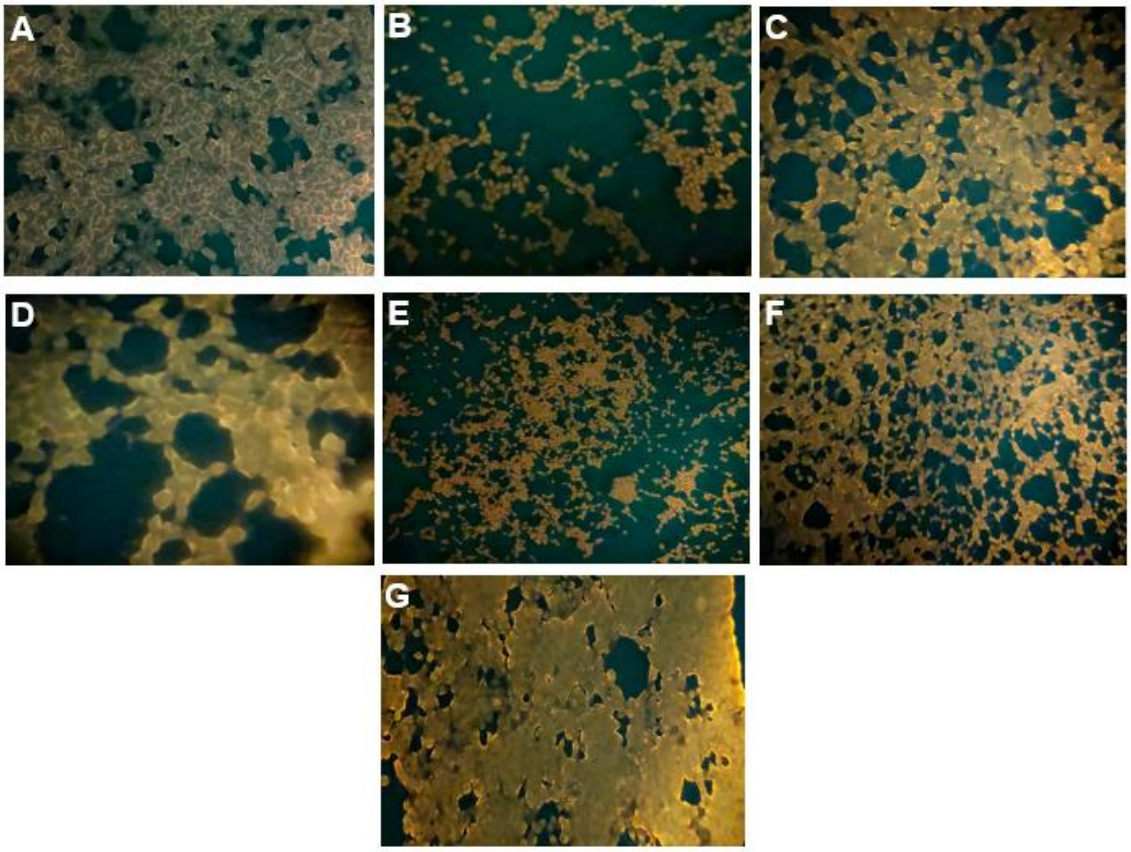

Fig 3 Fluorescent microscopic image of ECV304 cell line; control (A), treated with pinecone yoghurt at a concentration of 0,31% (vol/vol) (B), treated with pinecone yoghurt at a concentration of 0,63% (vol/vol) (C), treated with pinecone yoghurt at a concentration of 1,25% (vol/vol) (D), treated with pinecone yoghurt at a concentration of 2,50% (vol/vol) (E), treated with pinecone yoghurt at a concentration of 5% (vol/vol) (F), and treated with pinecone yoghurt at a concentration of 10% (vol/vol) (G) (Magnification 10X x 20X)

GC-MS analysis

The primary essential oils identified in yoghurt fermented with pinecone were hexadecanoic acid, eicosanoic acid, stearic acid, and 2-palmitoylglycerol. Table 1 shows the main compounds in the essential oil data in detail.

Table 1 The major essential oils in yoghurt fermented using pinecone

Compound	%
2-palmitoylglycerol	30,73
Stearic acid	24,11
Eicosanoic acid	21,69
Hexadecanoic acid	21,63
n-decane	0,78
4-hydroxybutanoic acid	0,36
Ethanimidic acid	0,29
Dimethylmalonic acid	0,15
Methylmalonic acid	0,14
Pentanoic acid	0,08
2-amino-butyric acid	0,04
Ethylmalonic acid	0,03

Discussion

The impact of fermented pinecone yoghurt on ECV304 cell cultures was examined in this study. Yoghurt was fermented using immature pinecone gathered in the spring and the effect of this fermented pinecone yoghurt on ECV304 cell cultures was investigated. The fermentation process is aided by two lactic acid bacteria found in the immature pinecone, *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*. The fermentation procedure utilizing pinecones was effective in producing yoghurt when the proper protocol was followed. Pinecones also contain lactic acid bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*, according to Bostan et al. (2017) [16]. After obtaining the extract of yoghurt fermented from a pinecone, selected six different doses from the study of Chen et al. (2007) Serial dilution was used to prepare these doses [0,31%, 0,63%, 1,25%, 2,50%, 5%, and 10% (vol/vol)] [19]. In this study,

using the ECV304 cell line, no doses other than these six doses were employed. All concentrations [0,31%, 0,63%, 1,25%, 2,50%, 5%, and 10% (vol/vol)] increased cell viability in the ECV304 cell line when compared to the control. The dose at 10% (vol/vol) concentration, which was predicted to have the greatest effect on cell viability, showed the expected findings. The effect of the 0,31% (vol/vol) dose, which was supposed to have the least amount of proliferation, did not turn out to be as predicted. The 0,63% (vol/vol) dose with the lowest proliferative impact and the 0,31% (vol/vol) dose, on the other hand, had no significant difference. In addition, MDA levels in the ECV304 cell line reduced at doses of 0,31%, 1,25%, 2,50%, and 10% (vol/vol) when compared to control. Since there is a deviation in 5% (vol/vol) concentration, the relevant dose has been omitted from the graph. The dose with the lowest MDA level is the dose at 1,25% (vol/vol) concentration. However, when based on the graph in general, it is obvious that each dose decreases MDA levels at roughly the same rate. The fact that MDA levels were lower in the pinecone fermented yoghurt group compared to the control group suggests that it possesses antioxidant capabilities. Finally, the findings of the previous MTT and LPO assays were verified by fluorescence microscopy images acquired following treatment with the ECV304 cell line at doses of 0,31%, 1,25%, 2,50%, and 10% (vol/vol). No harmful effects were seen in the cells when any of the doses were given. Acridine Orange/Ethidium Bromide staining revealed no apoptosis or necrosis in the cells. Although the dose that enhanced vitality the least in a cell viability test with the ECV304 cell line was 0,63% (vol/vol), the MDA level was similar to the other doses, and no apoptosis was seen in fluorescence microscopy. This finding is also supported by the healthy cell density observed in the fluorescent microscope and the cell viability test performed. As a consequence, different doses of pinecone yoghurt were shown to improve cell viability and decrease oxidative stress in the healthy cell line ECV304 in this investigation. The essential oils in yoghurt fermented with a pinecone and the essential oils in other fermented yoghurt kinds identified in the literature overlapped, according to the GC-MS data obtained in this study [22-23]. In the literature, the GC-FID method is also presented for the determination of fatty acids in commercial yoghurt [24]. Similar to our study, only stearic acid and hexadecanoic acid were found as major fatty acids in this study [24]. According to the literature research, there was a previous study that used pinecone for fermentation. Bostan et al. (2017) fermented yoghurt from a

pinecone, and in another study by Sert et al. (2017), *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* were identified in pinecones [16-25]. Aside from that, there was no other study that looked at any cell proliferation and antioxidant characteristics of pinecone fermented yoghurt in the literature. This study would be the first.

Conclusion

This study is believed to be a pioneering study as a result of this. An antimicrobial test may be done in the following phases of the study to see if pinecone yoghurt has an antibacterial impact, or a comparison of the two yoghurt can be undertaken using all of the characteristics determined in this study and a commercially purchased yoghurt. Based on the substantial results of the parameters done and the fact that the yoghurt developed in this study has no additives, athletes, pregnant women, children, and the elderly can consume it with confidence. Yoghurt fermented using pine cones has antioxidant properties and increases healthy cell vitality.

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Research Article

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Determination of *in vitro* Synergy of Ampicilin and Chloramphenicol against Multidrug Resistant *Bacillus cereus* Species

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ABSTRACT
Nowadays, combination therapy has become one of the most effective clinical practices in treating infections due to the emergence of multi-resistant microorganisms. In this study, minimum inhibitory concentrations (MICs) of six selected antibiotics; ampicillin, gentamicin, tetracycline, rifampicin, chloramphenicol, and ciprofloxacin were screened towards five *Bacillus cereus* isolates; KS2, E2, F2, F6, and K2W2 isolated from aquaculture sources and river in Kukup, Johor, Malaysia. Determination of MICs on tested antibiotics showed that all *B. cereus* isolates were resistant towards ampicillin and rifampicin but most sensitive to chloramphenicol, ciprofloxacin, and gentamicin. Apart from that, this investigation also provides the synergistic effect of ampicillin and chloramphenicol against the *B. cereus* isolates. On contrary, K2W2 resulted as an antagonism while F6 resulted as indifference. In particular, synergy or double therapy of antibiotics may be required to treat multi-resistant organisms. Furthermore, the observed synergy between ampicillin and chloramphenicol opens a new window of using bacteriocins and antibiotics in combination therapy of infections.

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Introduction

Antibiotics resistant (ABR) or antimicrobial drugs are defined as bacterial intrinsic resistance to certain antibiotics by undergoing mutations of the chromosomal gene as well as horizontal gene transfer [1]. Microorganisms can avoid being killed by antibiotics molecules through sophisticated mechanisms of drug resistance [2], and capable to survive the effect of antibiotic molecules due to the antibiotic's mechanism is no longer inhibiting their growth [3]. This probably happens because of the evolutionary changes in the resistance genes increasing the tolerance of antibiotics and leading to the situation whereas the antibiotic that used to wonder drugs is less functioning to fight off or to combat the infections [4]. The issues of ABR are clinically important as the antibiotics resistance genes can be horizontally transferred to human-associated bacteria and thus contribute to antibiotic resistance proliferation.

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Technically, ABR tends to spread from one microorganism to another or between species through the acquisition of a foreign gene or mobile gene via the process of horizontal gene transfer (HGT) [5]. Interestingly, food animals such as cattle, poultry, pigs, and aquaculture are also considered as a big contributor to bacterial resistance. The use of antibiotics in agriculture and aquaculture serves as a great concern in contributing to the emergence of ABR as they are widely used not only as a source of food but also considered as a source of income [6].

Researchers has found that problem arises on monotherapy to treat infections related to multidrug-resistant (MDR) as they are lesser in efficacy. Some challenges faced in Intensive Care Unit is having difficulty in achieving an adequate treatment for an infection caused by carbapenems producing bacteria and claimed that optimal efficacy was not achieved through monotherapy with agent like polymyxin [7]. Furthermore, the monotherapy antibiotics are not susceptible to certain bacteria particularly for antibiotic-producing bacteria. Some researchers reported that the mortality rates of patient with sepsis or septic shock treated with antimicrobial monotherapy does not decline and it is however increase the mortalility rates [8].

Previously, conventional foresight like antibiotics was once used as the best technique to fight bugs. Combination therapy is generally a plausible method and effective way to fight resistance compare to monotherapy. Combination therapy refers to two antibiotics that are synergistic or combine to produce a stronger effect compares to individual drugs alone [9]. However, there are some risk associated with the combination that are excessive for the antibiotics such as toxicity, costs, resistant strains selection and also superinfections [10]. Hence, the synergistic combination is relatively important for the treatment of MDR bacteria with lower does therapeutic effect.

Therefore, in this study, five *Bacillus cereus* isolates from the same strain that were isolated from rivers and fishes in Kukup area were selected, as Kukup involving aquaculture fishery activities, which may have become the reservoir for spreading of these bacterial genes to the water. This study was conducted for antibiotic susceptibility testing (AST) including synergistic test via checkerboard method to test for their antibacterial activity towards six selected antibiotics, with the hope that synergy or combination can be applied as an alternative antibacterial in the future.

Material and Methods

Materials

Five *Bacillus cereus* (KS2, K2W2, F2, F6, and E2) were obtained from previously cultured bacterial isolates in the form of glycerol stock(s) stored at -80℃ in Universiti Teknologi Malaysia (UTM). These bacterial isolates were streaked respectively under sterile condition on nutrient agar (Oxoid) prior to performing antibiotic susceptibility test (AST). Next, all of the *B. cereus* isolates were suspended in Mueller Hinton Broth (MHB) via direct colony suspension method before the determination of minimum inhibitory concentrations (MICs) of different antibiotics were performed. All Thermo Fisher Scientific Oxoid Microbiology media were purchased from VNK Supply & Services, Johor, Malaysia. Six antibiotics with certificates of authentication were used including ampicillin, gentamicin, tetracycline, rifampicin, chloramphenicol, and ciprofloxacin and were purchased from Bio-Basic, Canada.

Sampling site profile

A series of serial dilution was prepared and the diluted sample was spread on the nutrient agar followed by bacterial incubation at 37°C for 24 hours. The isolated bacterial was characterized based on their morphological characteristics and Gram staining was performed [11]. The morphological characterisation and Gram staining were served as the preliminary data for phenotypic bacterial identification.

Antibiotic susceptibility testing

Aquaculture sources (fish) and water samples were collected from Kukup (1°19'40.0°N, 103°26'22.9°E). Water samples were collected from three points by using the dip sampling method and three fish samples were collected in each location. Fish samples were transported at 4 °C to the laboratory and dissected within 4 h after collection according to the standard operating protocol [12]. The sample processing was performed on the fish samples where 10 g of internal guts and digestive tracts were ground with 10 ml sterile distilled water and 1 ml aliquot volume was measured and homogenized in 9 ml of sterile distilled water to give a 1:10 dilution [13]. The collected fish sample is *Lates Calcarifer*.

Genotypic Identification of Bacterial Isolates via 16S rRNA Sequencing

The bacterial DNA was extracted from overnight culture by using the simple boiling method [13, 14]. The extracted DNA was used to perform PCR amplification with GOTAQ® Promega Green Master Mix and 0.5 μM forward primer (fD1, 5'-AGAGTTTGATCATGGCTCAG-'3) and reverse primer (rP1, 5'-ACGGTTACCTTGTTACGACTT-'3) [15]. The forward and reverse primer were

manufactured by Integrated DNA Technologies (IDT), Malaysia. The PCR mixtures (25 μL) method started with the preheating step for activation of *Taq* polymerase at 95 °C for 3 mins, followed by 30 cycles of denaturation at 95 °C for 40 sec, annealing process at 55 °C for 30 sec and extension at 72 °C for 1.5 min, with a final extension at 72 °C for 5 min [16]. The PCR amplicons were electrophoresed in 1% w/v agarose gels with a molecular size marker (1kb GeneRuler) at 85 V for 45 min. The gel was stained with ethidium bromide for 5 minutes, rinsed, and viewed under ultraviolet light illumination. The resulting band size of the amplicons was ~1500bp.

DNA Sequencing and Phylogenetic Analysis

The unpurified PCR products were outsourced to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for sequencing. The resulting DNA sequences were analyzed by using the Bioedit software (version 7.2.5.0) to obtain the complementary sequences. The sequences of the PCR products obtained were analyzed with nucleotide Basic Local Alignment Search Tool (BLASTn) and by multiple sequence alignment using the ClustalW program provided by the National Center of Biology Information (NCBI) [17]. The phylogenetic analysis was carried out by using MEGA 7 software (version 10.1.1) to generate the phylogenetic tree and the relationship between the isolates of the most abundant bacterial species in each site. The multiple sequence alignments were performed with ClustalW [18] and the phylogenetic tree for each site was constructed by MEGA 7 with 1000 bootstraps [19].

Antibiotic Stock Solution Preparation

Stock solution for six tested antibiotics includes gentamicin, ampicillin, tetracycline, chloramphenicol, ciprofloxacin, and rifampicin with potency were suspended in 10 mL suitable solvent or diluent as shown in Table 1. The stock solutions were then stored in the refrigerator at 4℃ for further analysis.

Table 1 Solvent for six different antibiotics include ampicillin, gentamicin, tetracycline, rifampicin, chloramphenicol, and ciprofloxacin stock solution

Antibiotic	Solvent/diluent	Storage	Brand Name/Manufacturer
Ampicillin	Sterile distilled water	4˚C	Bio Basic, Canada
Gentamicin	Sterile distilled water	4˚C	Bio Basic, Canada
Tetracycline	70% Ethanol	- 20 ˚C	Bio Basic, Canada
Rifampicin	Absolute Methanol	4˚C	Bio Basic, Canada
Chloramphenicol	Absolute Ethanol	4˚C	Bio Basic, Canada
Ciprofloxacin	Sterile distilled water	25 ˚C	Bio Basic, Canada

Determination of Minimal Inhibitory Concentrations (MICs)

An inoculum equal to a 0.5 McFarland turbidity standard was prepared from each *B. cereus* isolate, and 10µl of the suspension was inoculated onto Mueller-Hinton agar plates. The MICs of the tested antibiotics were determined by the broth microdilution method as described [20]. The MICs of ampicillin, ciprofloxacin, rifampicin, chloramphenicol, gentamicin, and tetracycline, for the 5 *B. cereus* isolates were determined by using 96 well microtiter plate (Eppendorf). Next, 2X higher stock is prepared for each antibiotic. The stock solutions were prepared by using the formula [21]:

$$\frac{1000}{P} \times V \times C = W$$

where P = potency given by manufacturer (µg/mg), V = volume required (ml), C = final solution concentration (multiples of 1000) (mg/l), and W = weight of antibiotic (mg) to be dissolved in volume V (ml). Then, 50µl MHB was added into each well include positive and negative control well. A total volume of 50ul of 2X higher of the final concentration of antibiotic is added into column 1. Two-fold serial dilution was performed by transferring 50ul from column 1 to column 10. The process was repeated for each column and each row for each antibiotic. Next, 10µl of bacterial suspension is added into the well except for Column 12 (sterility control). After 24 hours of incubation, 5ul of resazurin assay (6.75mg) were added to indicate the viability of the cell and the result for MICs were observed after 4 hours of incubation with resazurin. Columns with no colour change (resazurin remained as blue colour) will be taken as the MIC value [22].

Synergistic Testing (Checkerboard Assay)

The Checkerboard assay was used to evaluate synergism among ampicillin and chloramphenicol against the *B. cereus*. Broth microdilution assay was performed on a 96-microtitre plate. Each isolate was tested against double combinations of antibiotics. A double combination including ampicillin and chloramphenicol were chosen against *B. cereus*. A single (MIC test for chloramphenicol and ampicillin) and double combinations of antibiotics against a single isolate of *B. cereus* were performed on 96-well plates as described by Elshikh *et al*. (2016) with modifications [22]. Briefly, columns 1 and 2 were used for the determination of MICs for each antibiotic alone (MIC test for chloramphenicol and ampicillin). Two times higher of the final concentration of antibiotics was added and two-fold serial dilution were performed from row A to row H (columns 1 and 2). Column 11 and

column 12 were acted as a growth control and sterility control, respectively. Columns 3 to 10 were used for double antibiotics combination for chloramphenicol and ampicillin as described by El-Azizi (2016) with modifications [20]. Briefly, each well was filled with 50µl MHB. Fifty microliters of the ampicillin at 4x higher tested were added into column 3 (A3 to H3) and two-fold serial dilution was performed for each row. The remaining 50µl portions were discarded from each last row. Then, 50µl of the chloramphenicol at 2x higher tested was added into row A (A3 to A10) and a two-fold serial dilution was performed for each column (A to H). The remaining 50µl portions were discarded from every last column. Finally, a volume of 10ul bacterial suspensions was added into each well except for column 12. All plates were incubated at 37˚C for 24 hours. After 24 hours of incubation, 5ul of resazurin assay (6.75mg) were added to indicate the viability of the cell and the result for MICs for double antibiotic combination were observed after 4 hours incubation with resazurin. Columns with no colour change (resazurin remained as blue colour) will be taken as the MIC value [22]. The calculation for the Fractional inhibitory concentration index (FICI) was used to interpret the synergistic result [23].

$$FICI = \frac{MICs\ of\ drug\ A\ in\ combination}{MICs\ of\ drug\ A\ alone} + \frac{MICs\ of\ drug\ B\ in\ combination}{MICs\ of\ drug\ B\ alone}$$

Results and Discussion

Bacteria Identification

Five *Bacillus* species isolates were successfully isolated from fish and water samples from Kukup; KS2, F2, E2, F6, and K2W2. All of the isolates yielded amplicons with the expected band size of 1.5 kb with good intensity and brightness in gel analysis. High identity percentages (92-100%) to the *B. cereus* strain (accession number: NR_074540.1) and low E-values were observed in the BLASTn analysis, which strongly suggests the accuracy and reliability of the identification results [24]. The *Bacillus* spp. occurs in the highest percentage in the fish samples as they are commonly used as probiotics in aquaculture [25]. All *Bacillus* isolates were identified as the same strain, which is *Bacillus cereus* strain ATCC 14579 as showed in Table 2. All the sequences of *B. cereus* isolates obtained were deposited into the NCBI gene bank and the sequences accession number were shown in Table 2.

Table 2 List of 16s rRNA sequences of *B.* cereus and the accession numbers obtained

Isolates	Species	Identity	Accession	Sequence ID deposited in the NCBI gene bank
E2	*Bacillus cereus* strain ATCC 14579 16S ribosomal RNA (rrnA), partial sequence	100 %	NR_074540.1	MK294257
F2	*Bacillus cereus* strain ATCC 14579 16S ribosomal RNA (rrnA), partial sequence	100 %	NR_074540.1	MK294260
F6	*Bacillus cereus* strain ATCC 14579 16S ribosomal RNA (rrnA), partial sequence	100 %	NR_074540.1	MK294264
K2W2	*Bacillus cereus* strain ATCC 14579 16S ribosomal RNA (rrnA), partial sequence	100 %	NR_074540.1	MK294267
KS2	*Bacillus cereus* strain ATCC 14579 16S ribosomal RNA (rrnA), partial sequence	100 %	NR_074540.1	MK294268

Minimal inhibitory concentration (MIC)

The resistant patterns of six antibiotics for five *B. cereus* isolates (KS2, F2, E2, F6, and K2W2) were determined and the results are shown in Table 3. Generally, every *B. cereus* isolate shows different MICs towards each antibiotic. Study revealed that all *B. cereus* that were isolated from different sources, showed the 100 % resistance to ampicillin. Interestingly, Investigation on the prevalence and antimicrobial susceptibility pattern of bacteria isolated from water and fish species *Rastrineobola argentea* and *Oreochromis niloticusi* and revealed that both fish and river showed that all the isolates were resistant to beta-lactam antibiotic (ampicillin) [26].

Previous study also reported that *B. cereus* isolates showed resistance to beta-lactam antibiotics [27]. Therefore, *B. cereus* generally produces beta-lactamase and is uniformly resistant to beta-lactam antibiotics. In a previous study by Sukmarini *et al.* (2014), they stated that most *Bacillus spp.* were susceptible to non β-lactam antibiotics [28]. In our study, all *B. cereus* isolates can be concluded to produce β-lactamase enzyme since they were all resistant towards beta-lactam antibiotics. The mechanism of beta-lactam degradation can be seen through the enzymatic inactivation. In this group, bacteria producing beta-lactamase enzymes synthesized beta-lactam antibiotic therefore keep increase in number which is inactivates enzymes include chloramphenicol and erythromycin [29].

From this study, it can be seen that all *Bacillus* spp. were resistant to rifampicin (Table 3). According to Soren *et al.* (2015), rifampicin antibiotics cannot be considered as a standard treatment due to there is no breakpoint for the resistance [30]. Rifampicin inhibits bacterial

DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent for instance in Gram-positive bacteria and mycobacteria. Rifampicin acts on bacterial RNA polymerase by adhering to the pocket of bacterial RNA polymerase β subunit 29 within DNA or RNA, hence it could block the expression of bacterial genes. However, rifampicin antibiotics cannot work alone due to β subunit of bacterial RNA polymerase (RNAP) easily get mutated [31]. In fact, instead of monotherapy combination therapy is used to stop the development of resistance and to shorten the length of treatment.

According to Lahiri *et al.,* (2016), most resistance towards rifampicin are acquired through missense mutations that take place in the rifampicin binding site on the RNA polymerase subunit that is responsible for determination of rifampicin resistance (encoded by rpoB gene which is the 81 base pair region) [32]. This statement is also supported by Vogler *et al.,* (2002) where beta subunit of RNA polymerase gene mutation are commonly pointed as the cause for rifampicin resistance [33]. The result in this study corresponding with their finding in which the resistance among *Bacillus spp*. can be particularly seen towards rifampicin antibiotics. Similarly, findings by Dabbs *et al.,* (1995) presented 20 *Bacillus* strain that were able to inactivate rifampicin antibiotics which including *B. Cereus* [34]. Besides, finding by Park *et al*., (2020) shows that particular strain *B. cereus* was highly resistant towards rifampicin [27].

Besides that, E2 and F6 show high resistance towards tetracycline. On contrary, F2 and K2W2 show the isolates were sensitive to the tetracycline. However, only one isolate from Table 3 shows indifference towards the tetracycline. Different sources show different patterns of antimicrobial susceptibility. Tetracycline antibiotics are extensively used for human medicine and aquaculture. Therefore, the resistance was higher according to E2 and F6. This may be due to this type of *Bacillus spp.* coming from the aquatic environment. Our findings are in agreement with some study which shows that the increase of resistance take place along the rivers subject to urban or agricultural activities that end ups in the aquatic environment that cause the presence of tetracycline and tetracycline resistant bacteria that originate from wastewater and source of agricultural [35]. This outcome also corresponds with Shah *et al.,* (2012) where only isolates from Tanzania fish farming consist of tetracycline resistance genes which are *tetA*(A) and *tetA*(G) are found, in which there are no history of antibiotic usage found in the location [36]. However, the mechanism that responsible for the high densities of the resistant bacteria in aquatic environment remains unclear. The main mechanism responsible for the emergence of tetracycline-resistant is efflux energy-dependent, protection of the ribosomal of bacteria, and enzymatic inactivation of the tetracycline molecule. Notably,

Bacillus spp. carry either gene *tet(L)* or *tet(K)* on the plasmid and/ or on their chromosome. Besides, these genes are mobilized through the conjugative plasmid and distribute within populations other than these genes are involved to encode the efflux protein which pump the tetracycline and doxycycline out from the cells [37].

Furthermore, all *B. cereus* isolates were sensitive to ciprofloxacin, chloramphenicol, and gentamicin. Similarly, Weber *et al.,* (1988) also explained that most *B. cereus* isolates were susceptible to chloramphenicol, ciprofloxacin, and tetracycline [38]. The outcome also compatible with Naas *et al.*, (2018) reported that *B. cereus* mostly sensitive to gentamicin, ciprofloxacin, and also chloramphenicol [39]. Chloramphenicol is a well-known drug that plays a major role as therapeutic agents. Consistent with other studies, our findings also reveal that the *Bacillus cereus* from different sources is most susceptible to chloramphenicol. This may be due to the *Bacillus cereus* provides a mode of action which chloramphenicol causes a bacteriostatic effect by binding to the 50S ribosomal subunit and inhibiting the transpeptidation step in protein synthesis [40].

In conclusion, all the isolates show different antimicrobial sensitivity on the tested antibiotics including ampicillin, gentamicin, tetracycline, rifampicin, chloramphenicol, and ciprofloxacin. In our study, all *B. cereus* were resistant to the ampicillin and rifampicin antibiotics. On contrary, all *B. cereus* were sensitive to gentamicin, chloramphenicol, and ciprofloxacin. The variation of antimicrobial sensitivity prevalent depends on the difference in concentration of antibiotic agents, differences source of isolates, drug resistance transfer, and widespread misuse of antibiotic in field [41].

Table 3 Determination of MIC Tested Antibiotics Against *Bacillus cereus* Isolates

Isolates	Antimicrobial agents	MIC range (µg/mL)	MIC standard from CLSI (µg/mL)			MIC (µg/mL)	Interpretation
			Susceptible	Intermediate	Resistance		
KS2	GN	0.03125 to 16	≤4	8	≥16	0.5	S
	TET	0.25 to 128	≤4	8	≥16	8	I
	RIF	0.03125 to 16	N.A.	N.A.	N.A.	0.125	R
	AMP	4 to 2084	≤8	16	≥32	1024	R
	CHL	0.25 to 128	≤8	16	≥32	4	S
	CIP	0.03125 to 16	≤1	2	≥4	0.25	S
F2	GN	0.03125 to 16	≤4	8	≥16	0.025	S
	TET	0.25 to 128	N.A.	8	≥16	2	S
	RIF	0.03125 to 16	N.A.	N.A.	N.A.	<0.03	R

	AMP	4 to 2084	≤8	16	≥32	128	R
	CHL	0.25 to 128	≤8	16	≥32	2	S
	CIP	0.03125 to 16	≤1	2	≥4	0.0625	S
E2	GN	0.03125 to 16	≤4	8	≥16	0.5	S
	TET	0.25 to 128	≤4	8	≥16	32	R
	RIF	0.03125 to 16	N.A.	N.A.	N.A.	2	R
	AMP	4 to 2084	≤8	16	≥32	2084	R
	CHL	0.25 to 128	≤8	16	≥32	4	S
	CIP	0.03125 to 16	≤1	2	≥4	1	S
F6	GN	0.03125 to 16	≤4	8	≥16	0.0625	S
	TET	0.25 to 128	≤4	8	≥16	32	R
	RIF	0.03125 to 16	N.A.	N.A.	N.A.	0.125	R
	AMP	4 to 2084	≤8	16	≥32	2084	R
	CHL	0.25 to 128	≤8	16	≥32	2	S
	CIP	0.03125 to 16	≤1	2	≥4	0.5	S
K2W2	GN	0.03125 to 16	≤4	8	≥16	0.125	S
	TET	0.015625 to 8	≤4	8	≥16	0.125	S
	RIF	0.0039 to 2	N.A.	N.A.	N.A.	0.25	R
	AMP	0.03125 to 16	≤8	16	≥32	2	S
	CHL	0.015625 to 8	≤8	16	≥32	0.25	S
	CIP	0.03125 to 16	≤1	2	≥4	0.0625	S

Fractional inhibitory concentration index (FICI)

Based on Table 4, KS2, F2, and E2 showed synergistic effects with FICI (≤0.5) on the antibiotic combination tested (ampicillin + chloramphenicol). There is no previous study shows the synergistic effect for the combination of ampicillin and chloramphenicol for *B. cereus spp* and this could be a new finding in which further study will be needed. On contrary, *B. cereus* isolates K2W2 shows antagonism with FICI (≥2.0) when introduced with ampicillin and chloramphenicol. This is strongly supported by Manten and Terra (1964), which reported that antagonism was obtained when chloramphenicol and β lactam group antibiotics (penicillin) were applied [42]. Despite chloramphenicol and penicillin are not from the same group of antibiotics, yet the synergistic effect still cannot be obtained since the chloramphenicol is a bacteriostatic antibiotic (reversible stoppage) may partly or completely destroy the bactericidal actions of penicillin [42]. Back in 1981, Weeks *et al.,* conducted a

clinical study which uses double therapy antibiotics such as bactericidal and bacteriostatic, resulted in poor outcome compared to the monotherapy bactericidal [43]. Notably, B. cereus F6 shows indifference (not additive or synergistic) results when introduced with ampicillin and chloramphenicol. Similarly, this outcome is compatible with Cole *et al.*, (1979) saying that there is no antagonistic effect between chloramphenicol and ampicillin combination also, indifferent (not additive or synergistic) effects when ampicillin and chloramphenicol being introduced [44]. Table 5 shows the FICI interpretation range that were used in this study.

Table 4 Synergistic Effect of Ampicillin and Chloramphenicol Combination Against Five *B. Cereus* Isolates

Isolates	MIC Antibiotic A (CHL) (µg/mL)		MIC Antibiotic B (AMP) (µg/mL)		FICI	Interpretation
	Alone	In combination	Alone	In Combination		
KS2	2	1	2048	256	0.625	Synergy
F2	0.5	<0.0625	16	<4	0.375	Synergy
E2	4	<0.125	2048	<64	0.0625	Synergy
F6	1	0.5	128	128	1.5	Indifference
K2W2	0.03125	<0.0078125	0.25	<0.0625	2.75	Antagonism

Table 5 FICI interpretation range use in this study

FICI Interpretation Range	
FICI ≤ 0.5	Synergy
0.5 < FICI ≤ 1.0	additive
1.0 < FICI ≤ 2.0	Indifference
FICI ≥ 2.0	Antagonism

Conclusion

All of the *B. cereus* isolates (KS2, E2, F2, F6, and K2W2) showed difference antimicrobial sensitivity towards the tested antibiotics; ampicillin, gentamicin, tetracycline, rifampicin, chloramphenicol, and ciprofloxacin. All *B. cereus* isolates were resistant to the ampicillin and rifampicin. On contrary, all *B. cereus* were sensitive to the gentamicin, chloramphenicol, and ciprofloxacin. The variation of antimicrobial sensitivity prevalent depends on difference in concentration of antibiotic agents, differences source of isolates, drug resistance transfer, and wide spread misuse of antibiotic in field. Three isolates (KS2, F2, and E2) showed synergistic

effects toward the antibiotic combination. More studies are required to facilitate the understanding of results obtained including the mechanism and the potential of these combination therapy as this could serve as preliminary data and can be useful to support therapeutic decisions clinically in future.

Abbreviations

ABR: Antibiotic Resisrant; HGT: Horizontal gene transfer, MDR: Multidrug-resistant; ICU: Intensive care unit; AST: Antibiotic susceptibility testing; UTM: Universiti Teknologi Malaysiat; NA: Nutrient Agar; MHB: Mueller Hinton Broth; MICs: Basic Local Alignment Search Tool; NCBI: National Cancer of Biology Information; CLSI: Clinical and Laboratory Standards Institute; FICI: Fractional inhibitory concentration index; RNAP: Bacterial RNA polymerase

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Availability of data and material

Please contact the corresponding author for any data request.

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Araştırma makalesi / Research article

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Elmada kurşuni küfe neden olan *Botrytis cinerea*'ya karşı boraksın etkisi

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ÖZET

Botrytis cinerea'nın neden olduğu kurşuni küf, elmaların hasat sonrası en önemli hastalıklardan biridir. Bu çalışmada, boraks ($Na_2B_4O_7$) tuzunun *B. cinerea*'ya karşı etkinliği hem *in vitro* hem de *in vivo*'da değerlendirilmiştir. *In vitro* testler artan boraks konsantrasyonlarının *B. cinerea*'nın misel gelişimi, spor çimlenmesi ve çim tüpü uzaması üzerindeki engelleyici etkileri arasındaki farklılığın istatistiksel olarak önemli olduğunu göstermiştir ($P<0.05$). Ayrıca misel gelişimi, spor çimlenmesi ve çim tüpü uzaması sırasıyla %2.0, %1.0 ve %1.0 boraks konsantrasyonlarında tamamen engellenmiştir. Öte yandan boraksın %1.0 ve %2.0 konsantrasyonlarının fungus misel gelişimi üzerine engelleyici etkileri arasında hiçbir önemli farklılık gözlenmemiştir ($P<0.05$). Boraksın EC_{50}, MIC ve MFC değerleri sırasıyla %0.263, %1.0 ve %1.0 olarak belirlenmiştir. *In vivo* denemeler hem koruyucu hem de tedavi edici boraks uygulamalarının kurşuni küfün hastalık gelişimini kontrole kıyasla önemli oranda azalttığını göstermiştir ($P<0.05$). Genel olarak hastalık gelişimine karşı koruyucu uygulamaların tedavi edici uygulamalardan daha üstün olduğu ve %3.0 boraks konsantrasyonunda, her iki uygulamada hastalık gelişiminin sırasıyla %85.69 ve %63.16 oranında azaldığı tespit edilmiştir. Sonuç olarak, çalışma bulguları, elma kurşuni küfünü kontrol etmek için boraks tuzunun kullanılabileceğini göstermektedir.

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Malus × *domestica*, hasat sonu, kurşuni küf, sodyum tetraborat, alternatif mücadele

The effect of borax against *Botrytis cinerea*, the casual agent grey mold on apple

ABSTRACT

Grey mold caused by *Botrytis cinerea* is one of the most important post-harvest diseases of apples. In this study, the efficacy of borax ($Na_2B_4O_7$) salt against *B. cinerea* was evaluated in *in vitro* and *in vivo*. *In vitro* tests showed that the difference between the inhibitory effects of increasing borax concentrations on the mycelial growth, spore germination and germ tube elongation of *B. cinerea* was statistically significant ($P<0.05$). Moreover, mycelial growth, spore germination and germ tube elongation were completely inhibited at 2.0%, 1.0% and 1.0% borax concentrations, respectively. On the other hand, no significant difference was observed between the inhibitory effects of 1% and 2% concentrations of borax on the mycelial growth ($P<0.05$). The EC_{50}, MIC and MFC values were 0.263%, 1.0% and 1.0%, respectively. *In vivo* experiments have shown that both protective and curative borax applications significantly reduce the disease development of grey mold compared to control. In general, it was determined that the protective applications against the disease development were superior to the curative applications, and the disease development was reduced by 85.69% and 63.16%, respectively, for both applications at 3% borax concentration. In conclusion, the study findings suggest that borax salt can be used to control apple grey mold.

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Giriş

Elma (*Malus* × *domestica* Borkh.) Kırgızistan'da yetiştirilen önemli meyve türlerinden biri olup, üretim miktarı bakımından 27.437 ha alanda yıllık 151.207 ton ile ilk sırada yer almaktadır [7]. Kırgızistan'da elma üretimi tüm bölgelerde yapılmasına rağmen, en fazla üretim Isık-Göl, Osh ve Çüy bölgelerinde yapılmaktadır [2].

Tarımsal ürünlerde hasat öncesi görülen hastalıkların yanında, hasat sonrasında biyotik ve abiyotik faktörlerin neden olduğu hastalıklar ürünlerin hasadı, paketlenmesi, taşınması ve depolanması aşamalarında ortaya çıkabilmektedir. Sonuçta depolanan ürüne, çeşidine ve depo koşullarına bağlı olarak %20-50 düzeyinde kayıplar görülebilmektedir [10]. Kırgızistan'da elma başta olmak üzere diğer önemli tarımsal ürünler için modern depolama ünitelerinin yaygın olmamasından dolayı depolanan ürünlerdeki kayıpların daha fazla olduğu bilinmektedir. Ürünlerin depolanması sürecinde özellikle fungal patojenlerin neden olduğu kayıplar son derece önemlidir. Elmalarda hasat sonrasında ortaya çıkan kayıplarda başta *Botrytis cinerea* ve *Penicillium expansum* olmak üzere *Monilinia fructicola, Glomerella cingulata, Alternaria* spp., *Rhizopus stolonifer* ve *Mucor pyriformis* türleri de ciddi ürün kayıplarına neden olabilmektedir [20]. Kurşuni küf etmeni *B. cinerea* üzüm, çilek, elma, kivi, domates, hıyar, biber ve süs bitkileri gibi 200'den fazla konukçu bitki türünün çiçek, yaprak, gövde, meyve ve diğer kısımlarına saldırarak hasat öncesi ve sonrası hastalıklara neden olmaktadır [5].

Dünyada hasat sonrası depolanan bazı ürünlerde fungal patojenlerin neden olduğu hastalıklara karşı sentetik fungisitler kullanılabilmektedir [29]. Ancak fungisitlerin insan ve çevre sağlığı üzerindeki olumsuz etkileri, bunlara karşı toplumsal farkındalığın oluşması ve *B. cinerea* gibi bazı patojenlerde fungisitlere karşı dayanıklılık probleminin ortaya çıkmasından dolayı, kimyasal mücadeleye alternatif mücadele yöntemlerinin geliştirilmesi ihtiyacı ortaya çıkmıştır [26]. Şimdiye kadar hasat sonu hastalıkların mücadelesinde fungisitlere alternatif olarak; biyolojik mücadele, sıcaklık uygulaması, organik, inorganik ve bazı bor tuz bileşiklerinin kullanımı, modifiye atmosfer ve ışınlama uygulamalarının tekli veya birlikte kullanım olanakları üzerinde çok sayıda çalışma yapılmıştır [4; 27; 14; 16; 3; 6; 28].

Bazı bor tuz bileşiklerinin tarımsal üretim alanlarında gübre olarak kullanımının yanında, hasat sonu depolanan tarımsal ürünlerde önemli kayıplara neden olan bazı fungal patojen (*B. cinerea, Penicillium digitatum, P. italicum, P. expansum, M. fructigena* vb.)'lere karşı

antifungal etkileri yapılan değişik çalışmalarda tespit edilmiştir. Bu tuzlar fungal patojenlerin hücre zar yapısına zarar verip, sitoplazma içeriğinin boşalmasına ve sonuç olarak ölüme neden olmaktadır [15; 19]. Qin ve ark. [16] potasyum tetraboratın farklı konsantrasyonlarda üzümde *B. cinerea'*nın misel gelişimini (%1.0), spor çimlenmesini (%0.5) ve çim tüpü oluşumunu (%1.0) tamamen engellediğini tespit etmişlerdir. Ayrıca bor uygulamasından sonra meyvelerde herhangi bir fitotoksisite rapor edilmemiştir. Benzer bir çalışmada *Colletotrichum gloeosporioides*'e karşı potasyum tetraboratın 20 mM konsantrasyonunun etmenin spor çimlenmesini %72.4 düzeyinde ve mango meyveleri üzerinde lezyon oluşumunun ise %46.1 düzeyinde engellendiği tespit edilmiştir [19]. Erper ve ark. [2019], disodyum oktaborat tetrahidrat (etidot-67) ve sodyum tetraborat dekahidrat (boraks dekahidrat)'ın *P. expansum*'un misel gelişmesini, spor çimlenmesini ve çim tüpü uzamasını güçlü bir şekilde engellediğini, bu etkinin tuzların artan konsantrasyonları ile ilişkili olduğunu bulmuşlardır. Ayrıca tedavi edici uygulamalarda, her iki tuzun %3.0 konsantrasyonunda elma meyveleri üzerindeki lezyon alanını kontrol uygulamasına göre sırasıyla %92.8 ve %78.9'a kadar, koruyucu uygulamalarda ise sırasıyla %94.3 ve %98.3'e kadar azalttığı ($P<0.05$) belirlenmiştir.

Kırgızistan'da hasat sonu elma gibi depolanan önemli tarımsal ürünlerde görülen fungal hastalıkların mücadelesinde, özellikle fungisitlere alternatif olarak kullanılabilecek bor tuzlarının etkinliklerinin belirlenmesi üzerine yapılan bir çalışma bildiğimiz kadarıyla bulunmamaktadır. Bu çalışmada boraks (sodyum tetrborat)'ın *B. cinerea*'nın misel gelişimi, spor çimlenmesi ve çim tüp uzamasına karşı etkileri ile fungitoksik etkileri *in vitro*'da belirlenmiştir. Ayrıca, elma meyveleri üzerinde kurşuni küf gelişimi üzerinde boraksın koruyucu ve tedavi edici etkileri de değerlendirilmiştir.

Materyal ve Metot

Fungal İzolat

Çalışmada kullanılan *B. cinerea* izolatı Kırgızistan Türkiye Manas Üniversitesi, Ziraat Fakültesi, Fitopatoloji laboratuvarındaki fungal kültür koleksiyonundan temin edilmiştir. Çalışmaya başlamadan önce, patates dekstroz agar (PDA; Merck Ltd., Darmstadt, Germany) besi ortamında geliştirilen kültürden hazırlanan inokulum (1×10^5 konidi mL^{-1}) sağlıklı elma meyve (cv. Grany Smith)'lerine bulaştırılarak izolatın virülensliği doğrulanmıştır. İzolat gelecek çalışmalarda kullanmak için PDA içeren eğik tüplerde +4°C'de saklanmıştır.

Bor tuzu

Çalışmada kullanılan boraks [sodyum tetraborat ($Na_2B_4O_7$)] Türkiye Eti Maden İşletmeleri Genel Müdürlüğü'nden temin edilmiştir.

Misel gelişimi üzerine boraksın etkisi

Boraksın *B. cinerea*'nın misel gelişimi üzerine etkisi Türkkan ve Erper [7]'in metoduna göre değerlendirilmiştir. Çalışmada kullanılan boraksın 7 farklı konsantrasyon (%0.0312, 0.0625, 0.125, 0.25, 0.5, 1.0 ve 2.0, w/v)'u sterilize edilmiş ve 50-55°C'deki PDA besin ortamına eklenmiştir. Boraksın tüm besin ortamına homojen bir şekilde karışması için magnetik karıştırıcı kullanılmış ve farklı konsantrasyonları içeren PDA besin ortamı, steril Petri kaplarına (6 cm çaplı) 10 ml olacak şekilde dökülmüş ve her bir konsantrasyonun pH'sı ölçülmüştür. Daha sonra PDA besin ortamında geliştirilmiş 5-7 günlük fungus kültüründen alınan 4 mm çaplı agar diskleri Petri kaplarına konmuş ve streç film ile çevrildikten sonra 20±1°C'deki inkübatör (TK 252, Nüve, Türkiye)'de inkübasyona bırakılmıştır. Aynı şartlarda inkübe edilen ve herhangi bir kimyasal uygulanmamış kontrol grubu Petri kaplarındaki fungusun misel gelişimi günlük olarak takip edilmiş ve kenara temas etmeden önce kontrol ve tuz konsantrasyonlarını içeren kaplardaki fungusun en uzun ve en kısa radyal gelişimleri ölçülmüştür. Fungusun misel gelişiminin engellenmesi MGE (%) = (kontrol Petri kaplarındaki fungal gelişme – tuz eklenmiş Petri kaplarındaki fungal gelişme / kontrol Petri kaplarındaki fungal gelişme) × 100 formülü kullanılarak hesaplanmıştır [12]. Deneme tuzun her bir konsantrasyonu için üç tekerrürlü olacak şekilde iki defa yapılmıştır [25].

Konidi çimlenmesi ve çim tüp uzunluğu üzerine boraksın etkisi

Botrytis cinerea izolatı PDA besin ortamında 20±1°C'de 5-7 gün geliştirilmiş, daha sonra üzerine steril saf su eklenmiş, steril bistirü yardımıyla konidiler suya geçirilmiş ve elde edilen süspansiyon 2 kat steril tülbentten geçirilmiştir. Süspansiyondaki konidiler Thoma lamı (hemocytometre; Marienfeld; Germany) kullanılarak konsantrasyonu 1×10^5 konidi mL^{-1}'ye ayarlanmıştır. Boraksın 7 farklı konsantrasyonu (%0.0312, 0.0625, 0.125, 0.25, 0.5, 1.0 ve 2.0, w/v) PDA'ya eklenmiş ve 6 cm çaplı Petri kaplarına dökülmüştür. Fungusun spor süspansiyonu (30µL) boraks eklenmiş ve eklenmemiş (kontrol) her kaba, steril cam baget ile yayılmıştır. Petri kapları 20±1°C'de 24 saat inkübasyona bırakılmış ve konidi çimlenme oranı (%), her konsantrasyonda 400 konidi olmak üzere, Euromex Delphi-X Observer (Arnhem, Holland) model mikroskopta 400× büyütmede

belirlenmiştir. İncelenen konidiler kendi büyüklüğü kadar çim tüpü oluşturmuş ise çimlenmiş olarak değerlendirilmiştir [6].

Ayrıca boraksın test edilen her konsantrasyonun çim tüpü uzaması üzerine etkisinin belirlenmesinde aynı kaplardaki 50 konidinin çim tüpü uzunlukları oküler mikrometre yardımıyla ölçülmüştür. Çim tüp uzunluğunun engellenmesi, ÇTUE (%)= (kontrol Petri kaplarındaki çim tüpü uzunluğu - tuz eklenmiş Petri kaplarındaki çim tüpü uzunluğu) / kontrol Petri kaplarındaki çim tüpü uzunluğu) x 100 formülüne göre hesaplanmıştır. Deneme tuzun her bir konsantrasyonu için üç tekerrürlü olacak şekilde iki defa yapılmıştır [6].

Misel gelişimi üzerine pH'nın etkisi

Fungusun geliştirileceği PDA besi ortamı steril edildikten sonra 50-55°C'ye kadar soğutulmuş ve besi ortamının pH'sı 1N sodyum hikroksit (NaOH) ve hidroklorik asit (HCl) ile 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 ve 13.0'a ayarlanmıştır. Daha sonra 6 cm çaplı Petri kaplarına dökülmüş ve bu kaplara PDA'da geliştirilmiş 5-7 günlük fungal kültürden alınan 4 mm çaplı agar diskleri inokule edilmiştir. Petri kapları 20±1°C'de inkübasyona bırakılmış, gelişmeleri günlük olarak takip edilmiş ve farklı pH'ları içeren kaplardaki fungusun en kısa ve en uzun radyal gelişmeleri ölçülmüştür. Deneme tuzun her bir konsantrasyonu için üç tekerrürlü olacak şekilde iki defa yapılmıştır [24].

Boraksın toksik etkisi

Fungusun misel gelişimini %50 engelleyen konsantrasyon (EC_{50}) değeri IBM SPSS İstatistik paket programı kullanılarak probit analizi ile belirlenmiştir. Ayrıca misel gelişimini tamamen engelleyen en küçük konsantrasyon (MIC= minimum engelleyici konsantrasyon) da belirlenmiştir [24]. Bunun yanında boraksın fungisidal/fungitoksik etkilerinin belirlenmesi için Thompson [22] ve Tripathi ve ark. [23]'nin metodları kullanılmıştır. Buna göre tuz konsantrasyonunu içeren Petri kaplarında gelişme göstermeyen fungal diskler alınarak, taze PDA içeren kaplara tekrar aşılanmış ve 20±1°C'de 9 gün boyunca takip edilmiştir. Bu süreçte fungal disklerde hiç bir gelişme gözlenmeyen konsantrasyon minimum fungisidal konsantrasyon (MFC) olarak değerlendirilmiştir.

Boraksın koruyucu ve tedavi edici etkileri

Boraksın 5 farklı konsantrasyonu (%1.0, 1.5, 2.0, 2.5 ve 3.0, w/v)'nun *B. cinerea*'ya karşı koruyucu ve tedavi edici etkileri elma meyveleri üzerinde belirlenmiştir.

Koruyucu uygulamalar için, kullanılan sağlıklı elma (cv. Grany Smith) meyveleri musluk suyu altında yıkanmış ve steril kabin içinde kurumaya bırakılmıştır. Yüzeysel dezenfeksiyon amacıyla meyveler %1'lik NaOCl'de 3 dk tutulmuş ve 2 kez steril saf sudan geçirilmiş ve kurutulduktan sonra dezenfekte edilmiş plastik viyollere yerleştirilmiştir. Elmaların ekvator bölgesine karşılıklı olarak 3 mm çaplı steril bir tel yardımıyla 3-4 mm derinlikte iki adet yara açılmış ve meyveler steril kabin içinde iki saat tutulmuştur. Daha sonra bu yaralara hazırlanan tuz konsantrasyonlarından alınan 25 µL'lik miktar mikropipetle yaralara uygulanmış ve bundan 2 saat sonra da 25 µL'lik %0.03'lük Tween 20 içeren 1×10^5 konidi mL^{-1} spor süspansiyonu tuz uygulaması yapılmış yaralara eklenmiştir.

Boraksın tedavi edici etkisinin tespitinde ise, önce 25 µL spor süspansiyonu (1×10^5 konidi mL^{-1}) mikropipetle aynı şekilde meyve üzerinde açılan yaralara uygulanmış ve 24 saat inkübasyona bırakılmıştır. Bu süre sonunda yaralara aynı tuz konsantrasyonları 25 µL olarak eklenmiştir. Her iki uygulamada negatif kontrol için meyvelerde açılan yaralara aynı miktarda %0.03'lük Tween 20 içeren steril saf su, pozitif kontrol için ise aynı miktarda fungusun spor süspansiyonu uygulanmıştır. Uygulama görmüş elmalar plastik kaplara yerleştirilerek inkübatörde 20±1°C'de inkübasyona bırakılmıştır. İnokülasyondan yedi gün sonra enfekteli meyveler üzerinde gelişen lezyon alanı belirlenmiştir. Bu amaçla lezyonlu alan üzerine yerleştirilen asetat kağıdına fungal gelişmenin sınırları çizilmiş ve bunlar 5 cm'lik bar olan beyaz A4 kağıtlarına aktarılmıştır. Bu çizimler Mustek 1200 UB Plus (Mustek Systems, Inc., Hsin Chu, Taiwan, PRC), masaüstü scanner ile taranmış ve Digimizer programı (Version 4.0.0.0 for Windows 2005-2011 MedCalc Software, Mariakerke, Belçika) ile yüzey alanları hesaplanmıştır. Denemeler üç tekerrürlü olarak yürütülmüş, her bir elma meyvesi bir tekerrür olarak kabul edilmiştir [26].

İstatistik analiz

Çalışmada elde edilen verilere IBM SPSS İstatistik programı (version 21, Property of SPSS, Inc.;IBM Company) kullanılarak ayrı ayrı tek yönlü varyans analizi (ANOVA) uygulanmış ve ortalamalar arasındaki önemli farklılıklar Tukey-HSD testi ($P<0.05$) ile belirlenmiştir.

Bulgular

In vitro uygulamalar *B. cinerea*'nın misel gelişimi üzerine boraksın farklı konsantrasyonlarının engelleyici etkileri arasında önemli farklılıklar olduğunu göstermiştir ($P<0.05$). En düşük 2 konsantrasyon (%0.0312 ve %0.0625)'da herhangi bir engelleme görülmezken, %0.125 ve üst dozlarda kullanılan tuz konsantrasyonları arttıkça patojenin misel gelişimi üzerindeki engelleyici etkilerinin de arttığı gözlenmiştir. Fungusun misel gelişimi üzerinde en yüksek iki konsantrasyon (%1.0 ve %2.0)'nun neden olduğu engellemenin diğer tüm konsantrasyonlardan istatistiksel olarak önemli ölçüde farklı olduğu tespit edilmiştir ($P<0.05$). Ayrıca tuz konsantrasyonu arttıkça besi ortamının pH'sının arttığı ve daha bazik hale geldiği, ancak bu artışın misel gelişimini engellemede çok önemli olmadığı görülmüştür (Tablo 1).

Tablo 1 Boraksın artan konsantrasyonlarının *Botrytis cinerea*'nın misel gelişimi, konidi çimlenmesi ve çim tüp uzunluğu üzerine engelleyici etkisi (%)

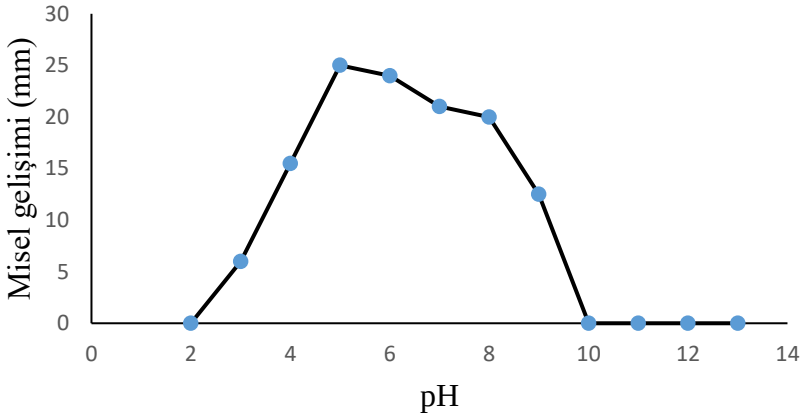
Table 1 The inhibitory effect of increasing concentrations of borax on mycelial growth, conidial germination and germ tube elongation of *Botrytis cinerea* (%)

Konsantrasyon (%, w/v)	pH	Engelleme (%)		
		Misel gelişimi	Konidi çimlenmesi	Çim tüp uzunluğu
0.0312	6.98	0.00 e	1.25 e	59.67 f
0.0625	7.45	0.00 e	4.25 e	69.81 e
0.125	7.95	17.86 d	24.00 d	73.70 d
0.25	8.46	57.14 c	40.50 c	80.88 c
0.5	8.60	71.43 b	61.00 b	87.19 b
1.0	8.78	97.62 a	100.00 a	100.00 a
2.0	9.04	100.00 a	100.00 a	100.00 a
Kontrol	5.70	0.00 e	0.00 e	0.00 g

*Aynı sütunda yer alan ve aynı harfle gösterilen ortalamalar arasında Tukey-HSD testine göre fark yoktur ($P<0.05$).

Boraksın artan konsantrasyonları ile fungusun konidi çimlenmesi ve çim tüp uzunluğu arasında engelleme yönünden pozitif bir ilişki olduğu tespit edilmiştir. Boraksın en düşük 2 dozunda fungusun konidi çimlenmesi üzerine engelleme oranı oldukça düşük kalmış ve kontrol uygulaması ile istatistiki olarak aynı grupta yer almışlardır. Buna karşılık boraksın %1.0 ve 2.0 konsantrasyonlarında konidi çimlenmesinin tamamen engellendiği ve bunun diğer konsantrasyonlara göre istatistiksel olarak önemli ölçüde farklı olduğu belirlenmiştir ($P<0.05$) (Tablo 1). Bununla birlikte boraksa karşı *B. cinerea*'nın çim tüp uzamasının konidi çimlenmesine göre daha hassas olduğu, diğer bir ifade ile boraksın en

düşük konsantrasyon (%0.0312)'unda konidi çimlenmesin %1.25 oranında, aynı konsantrasyonda çim tüp uzunluğunun ise yaklaşık %60 oranında engellendiği tespit edilmiştir (Tablo 1). Çalışmada ayrıca fungusun misel gelişimi üzerine 12 farklı pH'nın etkisinin değiştiği görülmüştür. En düşük pH (pH 2)'da ve pH 10-13'de misel gelişiminin tamamen engellendiği, buna karşılık fungus için en uygun pH değerinin pH 5-6 arasında olduğu belirlenmiştir (Şekil 1).



Şekil 1 *Botrytis cinerea*'nın misel gelişimi üzerine farklı pH'ların etkisi
Fig 1 Effect of different pHs on mycelial growth of *Botrytis cinerea*

Çalışmada test edilen boraksın *B. cinerea*'nın misel gelişimi üzerine toksik etkileri değerlendirildiğinde boraksın EC_{50} değerinin 0.263 (0.24-0.28) olduğu, bununla birlikte boraksın hem fungistatik konsantrasyon (MIC) değerinin, hem de fungisidal (fungitoksik) konsantrasyon (MFC) değerinin %2.0 olduğu tespit edilmiştir.

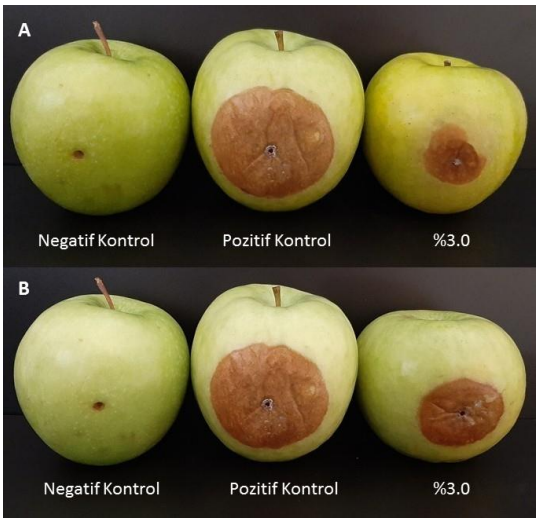
Boraksın artan konsantrasyonlarının *B. cinerea*'ya karşı koruyucu ve tedavi edici etkilerinin belirlenmesi amacıyla yapılan *in vivo* testlerde, boraksın meyve üzerindeki lezyon gelişimini kontrole göre önemli oranda azalttığı belirlenmiştir ($P<0.05$) (Tablo 2). Boraksın koruyucu uygulaması tedavi edici uygulamasına göre kurşuni küfü daha etkili bir şekilde kontrol etmiştir. Koruyucu uygulamanın en yüksek konsantrasyon (%3.0)'unda, elmalardaki lezyon gelişiminin kontrole göre yaklaşık %86 oranında engellendiği ve bunun istatistiki olarak önemli olduğu görülmüştür ($P<0.05$) (Tablo 2). Tedavi edici uygulamada ise tuzun aynı konsantrasyondaki lezyon oluşumunu engelleme oranı %63.16'da kalmıştır (Şekil 2).

Tablo 2 Boraksın artan konsantrasyonlarının elmada kurşuni küf etmeni *Botrytis cinerea*'ya karşı koruyucu ve tedavi edici etkileri (*in vivo*)

Table 2 Protective and curative effects of increasing concentrations of borax against the grey mould agent *Botrytis cinerea* on apple (*in vivo*)

Konsantrasyon (%, w/v)	Engelleme (%)	
	Koruyucu uygulama	Tedavi edici uygulama
1.0	15.64 d	20.12 d
1.5	34.04 c	30.18 c
2.0	45.20 cb	38.91 bc
2.5	54.16 b	45.36 b
3.0	85.69 a	63.16 a
Pozitif Kontrol	0.00 e	0.00 e

*Aynı sütunda yer alan ve aynı harfle gösterilen ortalamalar arasında Tukey-HSD testine göre fark yoktur ($P<0.05$).



Şekil 2 Boraksın kurşuni küf etmeni *Botrytis cinerea*'ya karşı %3.0'lık konsantrasyonda engelleyici etkisi, koruyucu uygulama (A), tedavi edici uygulama (B)

Fig 2 Inhibitory effect of borax against the grey mould agent *Botrytis cinerea* at a concentration of 3.0%, protective application (A), curative application (B)

Tartışma

Bu çalışmada önemli bir bor bileşiği olan sodyum tetraborat (boraks)'ın *B. cinerea*'nın neden olduğu elmada kurşuni küf hastalığının kontrolünde etkili olduğu belirlenmiştir. Antifungal etkinin boraksın artan konsantrasyonları ile arttığı görülmüştür.

Bitkilerin ihtiyaç duyduğu bir mikro besin elementi olan bor, bitkilerin kök uçları gibi aktif olarak büyüyen kısımları ile çiçek gelişimi, yaprak, tomurcuk, meyve ve tohum oluşumunda oldukça öneme sahiptir [1]. Ayrıca borun bazı formları orman endüstrisinde

ahşap malzemelerini bazı zararlı böcek ve funguslardan korumak amacıyla kullanılmış, yine sodyum tetraboratın iğne yapraklı orman ağaçlarında önemli patojenlerden bir olan *Heterobasidion annosum*'a karşı etkili olduğu tespit edilmiştir [9; 18]. Bunların yanında borun farklı formlarının önemli bazı bitki patojenlerine karşı da antifungal etki gösterdikleri yapılan farklı çalışmalarda tespit edilmiştir [17; 21; 3].

Çalışmada kullanılan boraksın konsantrasyonlarına bağlı olarak *B. cinerea*'nın misel gelişimi, konidi çimlenmesi ve çim tüp uzunluğu gelişiminin önemli oranda azaldığı ya da tamamen engellediği görülmüştür. Elde edilen bu sonuçlar borun farklı tuzlarının değişik patojenler üzerindeki antifungal etkilerinin tespiti amacıyla yapılan benzer çalışmalar ile uyumludur [16; 3; 11; 19; 6; 28]. Potasyum tetraboratın artan konsantrasyonlarının bağda kurşuni küf etmeni *B. cinerea*'nın misel gelişmesi, spor çimlenmesi ve çim tüp uzaması üzerinde engelleyici etkilerinin olduğu, bor tuzunun kontrol uygulamalarına göre %0.1'de konidi çimlenmesini ve %0.05'de ise çim tüp uzamasını önemli derecede azalttığı ($P<0.05$), %1.0'de misel gelişimini, %0.5'de ise spor çimlenmesini ve çim tüp uzamasını tamamen engellendiği rapor edilmiştir [16]. Benzer olarak Cao ve ark. [3] potasyum tetraboratın konsantrasyonu arttıkça *P. expansum*'un misel gelişiminin azaldığını, yani düşük konsantrasyonlarda (%0.01 ve 0.05, w/v) misel gelişimi gözlenmesine rağmen, %0.1'de %10-15'e kadar, %0.25'de ise tamamen engellemenin olduğunu tespit etmişlerdir. Yine aynı patojene karşı kullanılan etidot-67 ve boraks dekahidratın artan dozları ile engelleyici etkileri arasında pozitif bir ilişkinin olduğu, *P. expansum*'un misel gelişiminin %0.25'de, spor çimlenmesi ve çim tüp uzamasının ise %0.125'de tamamen engellediği belirlenmiştir [6]. Yapılan başka bir çalışmada borik asitin 6 farklı konsantrasyonunun elmada mavi küf etmeni *P. expansum*'un misel gelişimi, konidi çimlenmesi ve çim tüp uzunluğu üzerine antifungal etkisinin olduğu, %0.25 ve üst dozlarında %100 engellemenin olduğu tespit edilmiştir [28]. Patateslerde kuru çürüklük etmeni *F. sulphureum*'a karşı sodyum tetraborat ve potasyum tetraboratın konsantrasyonları arttıkça fungusun konidi çimlenmesinin ve misel gelişiminin azaldığı ve en yüksek konsantrasyon (20 g/L)'da ise tamamen engellenmenin gerçekleştiği rapor edilmiştir [11].

Çalışmada kullanılan boraks (sodyum tetraborat) en düşük konsantrasyon hariç, diğer üst konsantrasyonlarda alkali pH (7.45-9.04) göstermiştir. Boraksın pH'sı genel olarak 7.0'dan büyük olduğundan, antifungal etkinin alkali pH'dan kaynaklanıp

kaynaklanmadığı da bu çalışmada belirlenmiştir. Fungusun misel gelişimi üzerine tam engellemenin olduğu en üst konsantrasyon (%2.0)'da pH 9.04 iken, NaOH ile pH 9.0'a ayarlanan PDA besi ortamında fungusun misel gelişimi gözlenmiş, pH 10 ve üzerinde ise gelişme tamamen engellenmiştir. Elde ettiğimiz bu sonuç, fungusa karşı boraksın engelleyici etkisi üzerinde farklı pH'ların kısmen etkili olduğunu, diğer bazı organik ve inorganik tuzlar gibi bor bileşiklerinin pH'dan bağımsız olarak antifungal etki gösterdiğini ortaya koymuş olup, pH'nın engelleyici etkide küçük bir rolünün olduğunu bildiren önceki çalışmalarla da uyum içindedir [12; 13; 16; 24].

Bitki patojeni funguslara karşı farklı bor formlarının (bileşiklerinin), bazı organik ve inorganik tuzların bitki patojeni funguslara karşı toksik etkilerinin olduğu yapılan farklı çalışmalarda rapor edilmiştir. Kivide kurşuni küf etmeni *B. cinerea*'ya karşı kullanılan 6 farklı tuz arasında amonyum karbonat, amonyum bikarbonat ve sodyum karbonat tuzlarının EC_{50} değerinin 10 mM düşük olduğu ve MIC değerlerinin sırasıyla 25, 10 ve 25 mM olduğu rapor edilmiştir [26]. Etidot-67 ve boraks dekahidratın *P. expansum*'a karşı toksisitelerinin belirlenmesi için yapılan çalışmada EC_{50} değerlerinin sırasıyla 0.067 ve 0.071 olduğu belirlenmiş, minimum engelleyici konsantrasyon (MIC) değerleri %0.25 iken, aynı tuzların minimum fungisidal konsantrasyon (MFC) değerleri %1.0'dan büyük bulunmuştur [6]. Yapılan başka bir çalışmada aynı patojene karşı test edilen borik asitin EC_{50} değeri %0.09, MIC ve MFC değerleri sırasıyla, %0.25 ve >%2.0 olarak belirlenmiştir [28]. Li ve ark. [11], sodyum tetraborat ve potasyum tetraboratın *F. sulphureum*'un misel gelişimi üzerinde ED_{50} değerlerini sırasıyla 2.8 ve 3.2 g L^{-1}, konidi çimlenmesi üzerinde ise ED_{50} değerlerinin sırasıyla 2.1 ve 2.4 g L^{-1} olarak belirlemişlerdir. Çalışmada elde ettiğimiz sonuçlar daha önce yapılan çalışmalarla uyum içinde olup, boraksın *B. cinerea*'nın misel gelişimi üzerinde toksik etki gösterdiği ve EC_{50} değerinin 0.263 olduğu, ayrıca boraksın hem fungistatik konsantrasyon (MIC) değerinin hem de fungisidal (fungitoksik) konsantrasyon (MFC) değerinin %2.0 olduğu tespit edilmiştir. Çalışmada test edilen boraksın *in vitro* koşullarda etkili olan konsantrasyonlarının, *in vivo* koşullarda yapılan hem koruyucu hem de tedavi edici uygulamalarda etkili olmadıkları görülmüştür. Boraksın en yüksek iki konsantrasyonunda (%1.0 ve %2.0) misel gelişimi, konidi çimlenmesi ve çim tüp uzaması %97.62-100 oranında engellenirken, patojen inokulasyonunun 7. günü sonunda pozitif kontrol uygulamasına göre %1.0 ve %2.0 konsantrasyonlarda lezyon çapları sırasıyla, koruyucu

uygulamada %15.64 ve %45.20, tedavi edici uygulamada ise %20.12 ve %38.91 oranlarında engelleme görülmüştür. En yüksek konsantrasyon (%3.0)'da ise yaklaşık %86 ve %63 oranında engellemenin olduğu tespit edilmiştir. Daha önce yapılan benzer çalışmaların sonuçları bulgularımızı destekler nitelikte olup, antifungal etkiye sahip tuz bileşiklerinin *in vitro* ve *in vivo* etkinlikleri arasında bir pozitif ilişkinin olmadığı rapor edilmiştir [6; 28]. Bu uyumsuzluklarda konukçu doku-tuz interaksiyonları ve bazı çevresel faktörlerin etkili olduğu belirtilmektedir [8].

Sonuç olarak, bu çalışma ile boraksın *B. cinerea*'nın neden olduğu elmada kurşuni küf hastalığının mücadelesinde sentetik fungisitlere alternatif olarak kullanılabileceği tespit edilmiştir. *In vivo* koşullarda boraksın %3.0 konsantrasyonu uygulanan elma meyveleri üzerine herhangi bir fitotoksisite oluşmaması, bu tuzun hasat sonu hastalıklarına neden bitki patojenlerine karşı potansiyel olarak kullanılabileceğini göstermektedir. Boraksın fungusun hücre zarına verdiği zarar sonucunda sitoplazmik içeriğin dışarı çıkması ve sonuçta patojenin ölümüne neden olması şeklinde antifungal etkisinin olduğu bilinmektedir [16]. Bitkiler için gerekli bir mikro element olan borun farklı formlarının bitki patojeni fungus, bakteri, yabancı ot ve böceklerin mücadelesinde kullanılabilmektedir. Ayrıca başta elma olmak üzere hasat sonu depolanacak bazı ürünlerdeki fungal hastalıkların entegre mücadelesi için, boraks gibi antifungal etkiye sahip, bazı organik ve inorganik tuz birlikteliklerinin etkinliklerinin belirlenmesine yönelik çalışmalar yapılmalıdır.

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Research article

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The Investigation of Squeaky Feeling of Soap on the Skin

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ABSTRACT

The bar soap is one of the mostly used cleaning agents for centuries. After use of bar soap, there are some residues that cause a squeaky feeling which is favorable for some consumers because it reminds the feeling of cleanliness. However, some consumers do not like it. In this study, the reasons why the squeaky feeling is occurred after using the bar soaps were investigated. Through the ex vivo and in vivo tests, it was found that the residues on the skin were found due to magnesium and sodium salts of bar soap and magnesium and sodium ions in hard water. These result in the squeak and the amount of these salts and ions are affected by various parameters.

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Introduction

Although the bar soap is the oldest washing vehicle, the interaction of soap and kin has not been investigated sufficiently. It is observed that the squeaky feeling due to dryness and tension on the skin evokes the cleanliness, while some consumers do not prefer this. There are some arguments which propose that the soap removes the skin fat and dry the skin; and the residuals on the skin after soap use cause the squeaky feeling.

The external layer of skin stratum corneum is composed of proteins (65-75%), lipids (10-15%) and water (15%) and renewed by epidermis continuously [1, 2]. The capacity of skin barrier is related to the intracellular lipid molecular structure of stratum corneum [3].

When calcium and magnesium ions in bar soap contact with water, metal soap is formed. The solubility of the metal soap in water is very low so that it is hard to remove them from medium. Metal soap may remain between Stratum corneum layers [4,5].

Water hardness is a term used to describe the number of polyvalent ions found in water, especially the amount of calcium and magnesium. Calcium and magnesium bicarbonate salts $Ca(HCO_3)_2$ ve $Mg(HCO_3)_2$ cause "temporary hardness" or "carbonate hardness", while

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chlorine, sulfate, nitrate, phosphate and silicate salts of calcium and magnesium cause "permanent hardness". Hard water and soap can form a film called soap residue (white precipitate). The main component of these residues is calcium stearate from sodium stearate (soap) as in the following reaction [8].

The interaction of soaps and other surfactants with skin proteins cause skin barrier damage, redness, itching, the decrease in water holding capacity, penetration of surfactants and metal soaps into deeper layers of Stratum corneum because of protein swelling [6].

$$2C_{17}H_{35}COO^{-}{}_{(aq)} + Ca^{2+}{}_{(aq)} \rightarrow (C_{17}H_{35}COO)_2Ca_{(s)}$$

Studies have reported that the presence of calcium and magnesium salts in water increases the squeaky feeling on the skin (US 2013/0210696 A1) [9]. In another study, the squeaky feeling during rinsing was investigated by controlling the interaction between surfactant and cations. As a result, it has been found that the squeaky feeling in rinsing increases with the increase in water hardness (EP 1767186 A2) [10]. Similarly, in other studies, it has been reported that soap containing at least one anionic surfactant that can dissolve even in very soft water medium and a salt containing at least one multivalent cation is required for the formation of the squeaky feeling. The salt in the environment collapses on the skin surface with the surfactant and causes squeak (US 2013/0310296 A1) [11]. In addition, the degree of squeak can be increased by adding alkaline earth metal carbonates (calcium carbonate and magnesium carbonate) to soap-based products (US4557853) [12].

In the study of Conopco INC, D/B/A Unilever's patent number 2013/0210696 A1 published in 2012, tests were carried out with the Tribometer UMT device to increase the squeaky feeling left by cleaning compositions with calcium and magnesium salts and different surfactants. The friction test was performed by preparing solutions with certain concentrations with the solution, and it was observed that normal soap and synthetic surfactants had different levels of squeak as a result of the residues left on the skin at different rates.

In Patent no. 1767186A2 explains the compositions which give the increase in squeak during rinsing. The invention is also focused on the relationship between counter-ion and surfactant.

Specifically, it was found that increasing the precipitation of the counter ion surfactant complex helps reduce the micelle concentration of the surfactant, increase the surface tension, and increase the squeak.

In this study, the squeaky feeling after use of soap is investigated by using different tests for Evyap soaps which are produced as alkali soap by the saponification reaction.

Materials and Methods

In the study, two bar soap formulation were used with different fatty acid compositions as depicted in Table 1.

Table 1. Fatty acid compositions for bar soap formulations

Fatty acids	Toilet	Combo Soap
C12 (%)	10.45	3.14
C14 (%)	3.92	1.18
C16 (%)	40.97	12.30
C18 (%)	5.45	1.72
C18:1 (%)	27.93	8.34
C18:2 (%)	5.45	0.27
Glycerin (%)	0.90	0.31

Solution tests

A test was carried out to observe the interaction of soaps with different chemical structures with hard water. 2.3 gr $CaCl_2$/L was added to pure water and 15 FR hardness water was obtained. After adding 0.4 g of each bar soap formulation into hard water solution and they were mixed during two minutes and the water hardness values were measured again.

The effect of chelating agent on lime soap formation

The effect of chelating agent on lime soap formation was investigated by adding 1 gr Tetrasodium EDTA solution to 1% bar soap solutions dissolved in 15 FR water.

Testing on hydrophilic and hydrophobic silica surfaces

Internal tests were conducted to understand and examine the soap-skin interaction in more detail using hydrophilic and hydrophobic silica model surfaces. Stratum corneum is expected to show moderate surface energy like hydrophobic surface to see the effect of surface energy on the interaction of soap or lime soap with the surface.

Four different simulation methods were applied to prepare the silica surfaces. In first, silica surface was immersed into 5 ml of 0.1 w/w % Toilet soap dried with nitrogen. In second, silica surface was dipped in soap and then ultra pure water and dried with nitrogen. In third, surface layer was dipped in soap, then 40FR water and dried with N_2. In fourth, surface layer was dipped in soap, then adding $CaCl_2$ / $MgSO_4$ solution, then immersed in 40FR water and dried with N_2.

The effect of water hardness on residue on the skin - fluorescent dye test

Fluorescent dye has been used to visualize soap residues on the skin after washing and rinsing. The fluorescent dye has the tendency to bind with soap and can be homogeneously dispersed in soap-water solution. This method was tested by Wortzman M. (1986) [13] to evaluate the rinsing properties of 18 common soaps.

With reference to this method, a test was conducted on 10 panelists with the following parameters;

- 20 gr Toilet 1 soap was dissolved in 0 FR (Pure Water) and 60 FR hardness water.
- 0.05 gr Fluorescent dye (307027 Uranine-U, Sensient) was dissolved in soap solution
- A 5 cm diameter cloth moistened with a soap solution containing dye was applied to the panelists for 15 seconds
- The area where dyed with soap solution applied was rinsed with 250 ml tap water.
- Residue was photographed under UV light.
- Left arm was rinsed with 60 FR hard water, right arm was rinsed with pure water (0FR).

Result and Discussion

Interaction of soap's chemical structure with ions in water

It was observed that the water hardness decreased from 15 FR to 14.3 FR for Combo soap 9.8 for Toilet soap. It can be said that the carboxylate groups released by the dissolution of

the sodium salts in the two soaps bond with the Ca2 + ion and form a calcium salt, and this ratio is higher in Toilet 1 soap compared to Combo Soap as in Figure 1.

Fig 1 Interaction of soaps with ions in Water

The reason why the decrease of water hardness is high in toilet 1 soap is that its content is completely composed of sodium salts and the high rate of carboxylate group released by dissolving in water, bonds with Ca^{2+}; The reason why this ratio is low in mixed soap is that it contains very little sodium salt soap.

The effect of chelating agent on lime soap formation

In Figure 2, Solution A represents 1% Toilet 1 solution dissolved in 15 FR hard water, B indicates 1 g Tetrasodium EDTA solution added on 1% soap solution dissolved in 15 FR water. It was observed that after the soap dissolved, white precipitates formed in the water as in Solution A. When 1 gr Tetrasodium EDTA was added into the prepared solution, it was seen that the water became clear as in Solution B. It has been observed that Tetrasodium EDTA retains metal ions and therefore did not precipitate, resulting in clearer water compared to hard water with only soap added.

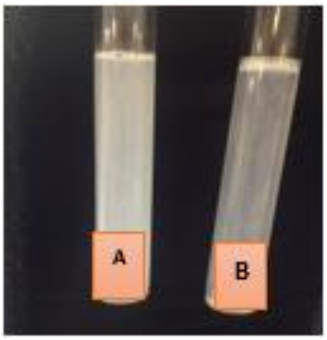

Fig 2 The effect of Tetrasodium EDTA on the precipitation of soap in hard water

Testing on hydrophilic and hydrophobic silica surfaces

The adsorption of precipitate on five hydrophilic silica surfaces was tested. The images in Figure 3 were obtained after the soap solution was applied to the surface. The dotted lines indicate the regions where sediment adsorption was observed, while the solid lines indicate the regions where adsorption was observed due to drying effects.

In Figure 3A, it was observed that when Toilet soap with 0.1% by mass is applied and dried with N_2, it formed a layer and remains on the surface after drying. In Figure 3B, it was observed that the layer formed quickly disappeared when MilliQ ultra pure water was rinsed with water due to the weak bond between the surface and soap. In Figure 3C and 3D, sediment adsorption was observed on the surface after rinsing with 40 FR water. Immediately after the addition of divalent Ca^{2+} and Mg^{2+} ionic electrolyte, the adsorption of the precipitates to the hydrophilic surface was observed. Adsorbed precipitates were low soluble Ca^{2+} and Mg^{2+} salts (lime soap). Electrons alone were observed to attach to the surface.

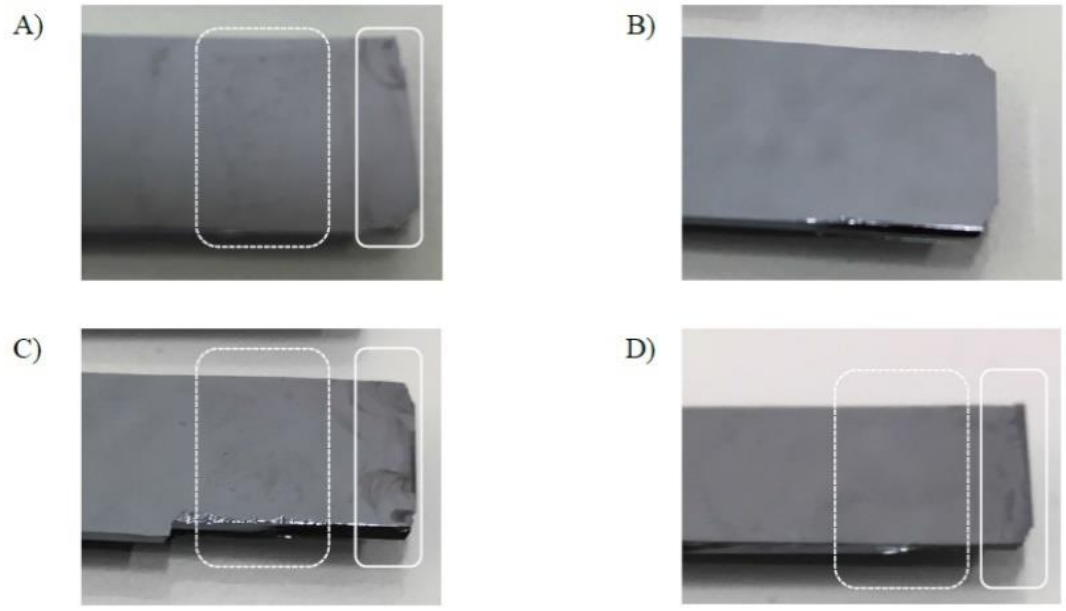

Fig 3 Images of hydrophilic model silica surfaces A) after immersion in soap and with N_2 After drying, B) first dipped in soap, then MilliQ water and set with N_2 C) first dipped in soap, then 40FR water and dried with N_2 D) first put in soap, then adding $CaCl_2$ / $MgSO_4$ solution, then immersed in 40FR water and dried with N_2

In the light of the studies and observations, soap and electrolytes alone do not hold on the surface for a long time, could be easily rinsed and leave no residue. However, it was concluded that Ca^{2+} and Mg^{2+} salts precipitated due to the electrolyte addition to the surface and did not move away.

After rinsing with 40 FR water and after electrolyte addition on hydrophilic and hydrophobic surfaces, soap residue and Ca^{2+} / Mg^{2+} salt deposits were formed in the same direction; but it was observed that the amount of precipitate formed after the addition of electrolyte on the hydrophobic surface was higher than the hydrophilic surface (Figure 4).

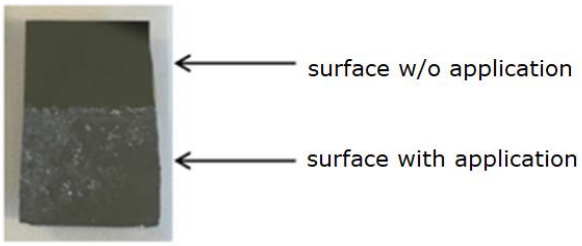

Fig 4 Hydrophobic Silica Model Surface - first immersion in soap, immediately after the addition of $CaCl_2$ / $MgSO_4$ solution, then immersion in 40FR water and drying with N_2

The upper surface of the Stratum Corneum is hydrophobic; more precipitation is observed on the hydrophobic silica surface than on the hydrophilic surface. After washing and rinsing with hard water, it was concluded that Ca^{2+}/Mg^{2+} salt precipitates in water could adhere to the skin.

Once bar soap contact with hard water, metal soap which main component is calcium stearate and magnesium stearate, is formed. The solubility of calcium and magnesium salts in water are very low so that it is hard to remove them from medium [4,8]. Due to presence of calcium and magnesium salts in water increases the squeaky feeling on the skin. Hence, It can be concluded that squeaky feeling in rinsing increases with the increase in water hardness [9,10,11].

Conclusion

In the study, it was concluded that when the surfactants formed as a result of the saponification reaction are dissolved in water. The free carboxyl groups compound with calcium and magnesium ions that determine the water hardness. When the soap is dissolved

in water, the water hardness decreases, and Tetrasodium EDTA prevents the precipitation in the soap solution. In panel tests, when Toilet soap and Combo soap were compared, it is found that soap caused squeak in hard water and no squeak was felt in soft water. There was no difference in the mixture of soaps in two different water hardness. The simulation studies were performed by considering the structure of Stratum Corneum on hydrophobic and hydrophobic surfaces and the soap accumulation was higher on the hydrophobic silica surface similar to the structure of the Stratum Corneum. Based on these results, it is intensely emphasized that the squeaky feeling may occur due to the precipitation of ions in hard water as calcium / magnesium salt on the skin.

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Research article

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In-silico functional annotation of a hypothetical protein from *Edwardsiella tarda* revealed Proline metabolism and apoptosis in fish

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ABSTRACT

Edwardsiella tarda is one of the most widespread pathogens in aquatic species. A wide variety of diseases can be caused by this microbe, including Edwardsiella septicaemia but clinical signs of infection differ between species of fish. The fact that the bacteria is resistant to a wide range of antimicrobials is extremely important. Furthermore, several proteins in its genome are classified as hypothetical proteins (HPs). As a result, the current work sought to elucidate the roles of an HP found in the genome of *E.tarda*. To determine the structure and function of this protein, many bioinformatics methods were used. To locate the homologous protein, the sequence similarity was searched across the available bioinformatics databases. Quality evaluation methods were used to predict and confirm the secondary and tertiary structure. Additionally, the active site and interacting proteins were examined using CASTp and the STRING server. An important biological activity of the HP is that it contains single functional domains that may be responsible for host-cell invasion and autolysis. Further, protein-protein interactions within selected HP revealed several functional partners that are essential for bacterial survival. One such partner is the proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase (putA) of *E. tarda*. In addition, molecular docking and simulation results showed stable bonding between HP and Proline metabolism protein. Finally, the current work shows that the annotated HP is associated with possible mitochondrial metabolism and autolysis formation activities, as well as having a stable binding with the putA protein, which might be of significant relevance to future bacterial genetics research.

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Introduction

Edwardsiella tarda (*E. tarda*) is found in a wide range of hosts, including humans, other animals, and fish. This is worth noting that *E. tarda*-related fish infections cause the most

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common bacterial illness in fish and can result in massive infection and mortality in both saline and fresh water. It may be found in a variety of aquatic ecosystems all around the world [1]. Edwardsiellosis and systemic hemorrhagic septicemia, both caused by *E. tarda*, were characterized by internal abscesses and extensive skin inflammation with coloration on fishes [2]. The catastrophic fatalities of farmed fish caused by *E. tarda* infection have resulted in significant financial damage in the United States of America, notably in Japan and India [3]. It seems to be worth noting that the infection in people usually results in dysentery, gastroenteritis, meningitis, delayed wound healing, and sometimes even mortality [4]. In addition to invasion capability, *E. tarda* can release siderophores implicated in induced inflammation and abnormal host cell function, to infect epithelial cells and form biofilms [5-8]. To minimize pathogenic bacterial burdens, tons of antibiotics have been thrown into pathogen-prone areas in recent years. Rather, this approach has worsened the threat posed to the aquaculture industry and public health by drug-resistant strains, such as *E. tarda* [9, 10]. Virulent *E. tarda*, identified from edwardsiellosis-infected Japanese flounder, has shown great resistance to kanamycin, tetracycline, ampicillin, and streptomycin. It also appeared to be resistant to a variety of other antibiotics often used in farmed fish [11]. To a large extent, tetracycline resistance is mediated mainly by proton-dependent efflux pumps, which are found in gram-negative bacteria, particularly *E.tarda*, as well as ribosomal proteins found in gram-positive bacteria [12]. Antibiotic resistance, on the other hand, is a complex phenomenon, with resistance mechanisms presumably involving complex networks of genes, proteins, and biological processes.

Because of the unique fimbriae it creates, *E. tarda* has a strong attraction for red blood cells. *E. tarda* exhibits hemagglutination characteristics as a result of this. The fimbriae are encoded in the genome as a 534-base-pair region [13]. Nevertheless, many of this bacterium's proteins are classified as HPs since their structures and biological activities are unknown. Such proteins can be extremely useful, and their annotation can lead to new insights into their structures, routes, and activities. Consequently, bioinformatics approaches can be utilized to predict and analyze various forms of the structure of those HPs, their biological functions, and their interactions with other proteins.

It got simpler to attribute function to an HP utilizing numerous bioinformatics methods as the in-silico study progressed. We aimed to develop a better understanding of the protein and further drug targets through the assignment of structural and biological functions to hypothetical protein AAW15_09260 (AKH89314.2) of *E.tarda*. Protein-protein interaction was investigated and subcellular distribution, secondary structure, and active site were predicted. In addition, homology modeling techniques were used to attempt to produce a good quality model of the AKH89314.2.

Methods

Identification of similarity and retrieval of sequences

The NCBI database was used to obtain the sequence information for the hypothetical protein (HP) (AKH89314.2). After that, the sequence was saved as a FASTA format and submitted to multiple prediction servers for in-silico analysis. Previously, scientists describe the possible role and effect of this selected HP in channel catfish (*Ictalurus punctatus*), however, they did not functionalize and characterize the HP [14]. Therefore, this HP of *Edwardsiella tarda* needs to be characterized to reveal its role in channel catfish. A similarity search was conducted with the NCBI protein database to provide a first prediction regarding the function of the targeted HP (https://www.ncbi.nlm.nih.gov/) against non-redundant [15] database to use the BLASTp tool to search proteins that may have similar characteristics to the HP [16].

Phylogeny analysis and multiple sequence alignment

Multiple sequence alignments were performed using the BioEdit biological sequence alignment editor between the HP and proteins with similar structural characteristics to the HP [17]. The phylogenetic analysis was performed using an older version of the Molecular Evolutionary Genetic Study (MEGA) (https://megasoftware.net/).

Physiochemical properties analysis

ExPASy's ProtParam (http://web.expasy.org/protparam/) tool was used to determine physical and chemical parameters such as molecular weight, amino acid composition, theoretical pI, instability index, extinction coefficient, atomic composition, estimated half-life, the total number of positively charged residues (Arg + Lys), the total number of negatively charged residues (Asp + Glu), aliphatic index, and grand average of hydropathicity (GRAVY) [18].

Subcellular localization analysis

CELLO anticipated subcellular localization [19]. The results were also compared to PSORTb subcellular localization predictions [20], PSLpred [21], and SOSUIGramN. TMHMM [22], HMMTOP [23], and CCTOP [24] were used for the topology prediction.

Identification of conserved domains, motifs, folds, families, and superfamilies

A search was conducted on the database of conserved domains (CDD, available at NCBI) [25], for the conserved domain. The Motif (Genome Net) server was used to find protein motifs [26]. The evolutionary connections of the protein were assigned using Pfam [27] and SuperFamily [28] database. For the functional analysis of the protein, the protein sequence analysis and classification software InterProScan [29] was used. The PFP-FunD SeqE server [30] was used to recognize protein folding patterns.

Prediction of secondary structure

The secondary structure of proteins was predicted using PSI-blast-based secondary structure prediction (PSIPRED) [31]. Its algorithm employs artificial neural networks and machine learning techniques. It is indeed a server-side application with a front-end website that can predict a protein's secondary structure (beta sheets, alpha helixes, and coils) based on its primary sequence.

3D structure prediction, Refinement, and Validation

The three-dimensional structure of the target protein was predicted using the Raptorx server (http://raptorx.uchicago.edu/) [32]. The protein's 3D structure was refined using GalaxyWeb. In homology modeling, which is based on empirically proven 3D protein structures, the structure's validity is a vital step. The suggested protein model was submitted to ProSA-web for basic confirmation. The z-score, which represents the overall character of the model, was predicted by the server. If the z-scores of the predicted model are outside the scale of the property for local proteins, the structure is incorrect. A Ramachandran plot analysis was performed utilizing the Ramachandran Plot Server to establish the overall quality of the protein (https://zlab.umassmed.edu/bu/rama/).

Assessment of model quality

Subsequently, the predicted three-dimensional structure was evaluated using PROCHECK, Verify3D, and ERRAT Structure Evaluation server.

Protein-Protein Interaction Analysis

Protein functions are determined by interactions between their residues. The STRING database (http://string-db.org/) was employed in this investigation, which analyzes physical and functional correlations to discover known and expected protein interactions. Genomic context, high-throughput investigations, (Conserved) Co-expression and prior knowledge were used to make this decision. This database quantitatively incorporates interaction data from the following sources [33].

Protein disulfide bonds

The formation of disulfide bonds between cysteine residues in a protein is critical for its folding into a functional and stable shape. CYSPRED and DIANNA were used to predict disulfide bonds within a hypothetical protein in order to get insight into the experimental structure determination and stability of the protein. CYSPRED evaluates whether your query protein's cysteine residues form disulfide bridges/bonds. CYSPRED is a neural network-based predictor that has been taught to accurately discriminate the bonding states of cysteine in proteins, beginning with the non-binding state of the residue chain [34]. DIANNA was also employed since it aids in the prediction of disulfide connections in a protein sequence input. Understanding the function of a hypothetical protein and tertiary prediction techniques rely heavily on the ability to accurately estimate disulfide bridges [35]. We will be able to identify docking sites for hypothetical proteins based on their tertiary structure, moving one step closer to creating drugs that target diseases caused by mutations in the hypothetical gene.

Ligand binding site prediction

To anticipate protein-ligand binding sites in hypothetical proteins, the Galaxy server was employed. GalaxySite predicts the ligand-binding site of a query protein based on its tertiary structure by protein-ligand docking. The structure may be either an experimental structure (with or without ligand) or a model structure. If a protein sequence is provided, GalaxySite predicts the structure by using the GalaxyTBM method without refinement step. The binding ligands are predicted from the complex structures of similar proteins detected by HHsearch.

The protein-ligand complex structures are then predicted by a ligand docking method called LigDockCSA [36].

Detecting active sites

This protein's active site was determined by using the Computed Atlas of Surface Topography of Proteins (CASTp), located at http://sts.bioengr.uic.edu/castp/ [37]. It is a web-based tool for identifying, defining, and quantifying concave surface areas on 3D protein structures.

Studies on molecular docking and simulation

The molecular docking and simulation studies were carried out using the ClusPro v2.0 server. The algorithm running behind the ClusPro v2.0 server is very robust and does not require any prior information regarding either template or binding site between the protein-protein. The online server can be accessed at (https://cluspro.bu.edu/). ClusPro provides a user-friendly interface for understanding flexible docking between protein-protein interactions [38]. A detailed insight into the docking procedure is provided by the server whilst providing complete flexibility to the peptide sequence as well as providing permissible flexibility to the protein receptor sequence. Finally, the docking prediction result, clustering details, and interaction models generated by ClusPro were analyzed. The final docked protein-protein complex was visualized in Discovery studio. Further, the amino acid interactions occurring between protein-protein complexes were tabulated using PDBsum [39]. HawkDock was used to calculate the binding energy of a protein-protein complex [40]. To estimate interaction free energies between diverse protein-protein complexes, the service uses molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) [40].

Result and Discussions

Similarity identification, Multiple sequence alignment, and phylogeny analysis

The results of BLASTp against a non-redundant database revealed similarities with other flagellum proteins (Tables 1). The FASTA sequences of the hypothetical protein (HP) (AKH89314.2) and homologous identified proteins were aligned using multiple sequence alignment (Supplementary Fig. 1). To corroborate homology assessments of proteins at the

complex and subunit levels, phylogenetic analysis was used. The alignment and BLAST results were used to create a phylogenetic tree, which offers a comparable idea about the protein (Fig. 1). The distances between branches are also taken into consideration.

Table 1. Non-redundant sequencing yielded a protein with similar properties

Protein ID	**Accession No. AKH89314.2**			
	Organism	**Protein Name**	**Identity (%)**	**e value**
WP_200900965.1	*Edwardsiella tarda*	hypothetical protein	100	0.00
WP_207769806.1	*Edwardsiella tarda*	hypothetical protein	99.78	0.00
WP_109579113.1	*Edwardsiella tarda*	sodium:solute symporter	98.90	0.00
WP_217700654.1	*Edwardsiella tarda*	hypothetical protein	98.46	0.00
WP_024524640.1	*Edwardsiella hoshinae*	sodium:solute symporter	95.18	0.00

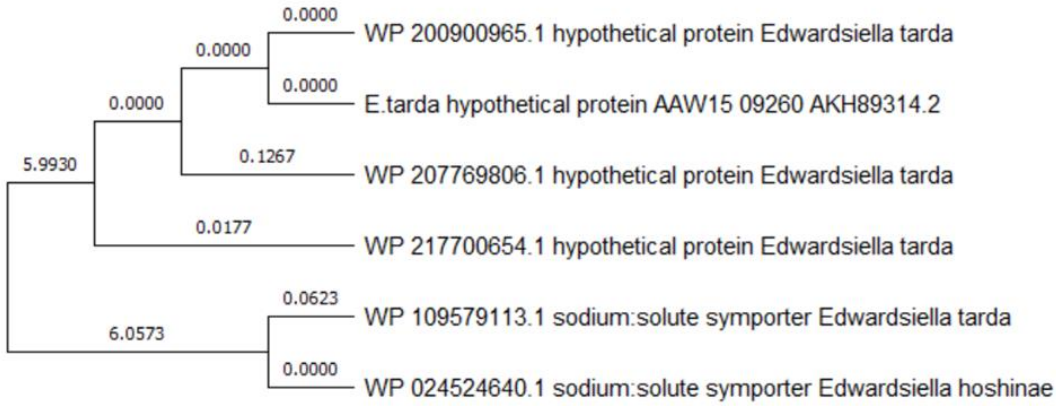


Fig 1 Multiple sequence phylogenetic tree

Physicochemical features

The protein consists of 384 amino acids, among the most abundant was Ala (A) 65 followed by, Leu (L) 58, Gly (G) 44, Ile (I) 40, Val (V) 38, Ser (S) 33, Phe (F) 26, Thr (T) 26, Arg (R) 19, Tyr (Y) 17, Pro (P) 15, Met (M) 13, Gln (Q) 13, Glu (E) 12, Trp (W) 11, Asn (N) 6, Asp (D) 6, His (H) 6, Lys (K) 5, and Lys (K) 5. The computed molecular weight was 48649.51 Da, with a theoretical pI of 9.22, indicating a positively charged protein. The total number of

positively charged (Arg + Lys) and negatively charged (Asp + Glu) residues were discovered to be 18 and 24, respectively. The protein was classified as unstable by the computed instability index of 35.49. The aliphatic index was 122.24, indicating that proteins are not stable across a wide temperature range. The GRAVY value was 0.899. GRAVY with a positive value implies that the protein is polar. Mammalian reticulocytes (in vitro) were found to have a half-life of 30 hours, yeast, > 20 hours, and Escherichia coli, > 10 hours. And the molecular formula of protein was identified as C2274H3544N560O588S16.

Table 2. Physiochemical features of hypothetical protein from different tools and server

ProtParam tool		**EMBOSS Pepstats**	
Sequence ID	AKH89314.2	Charge	9.0
Family (Pfam)	Sodium:solute symporter family	Improbability of expression in inclusion bodies	0.751
Domain (ScanProsite)	Sodium:solute symporter family	Average residue weight	106.688
Alignment	21–403	A280 extinction coefficients 1 mg ml-1	1.764
HMM length	383	A280 molar extinction coefficients	85830
Bit score	10.446	Tiny (A + C + G + S + T)	171
E value	3.3×10^{-112}	Small (A + B + C + D + G + N + P + S + T + V)	236
Number of AA	456	Aliphatic (A + I + L + V)	201
MW	48649.51 Da	Aromatic (F + H + W + Y)	60
pI	9.22	Non-polar (A + C + F + G + I + L + M + P + V + W + Y)	330
Extinction coefficients	42985	Polar (D + E + H + K + N + Q + R + S + T + Z)	126
Instability index	35.49	Charged (B + D + E + H + K + R + Z)	48
Aliphatic index	122.24	Basic (H + K + R)	30
GRAVY	0.899	Acidic (B + D + E + Z)	18

Hypothetical protein functional annotation

This potential protein sequence was discovered to have only a domain using the conserved domain search tool which is the SLC5-6-like_sbd superfamily (accession No. cl00456). Two further domain search tools, InterProScan and Pfam, were used to verify the result. Pfam

server predicted the Sodium: solute symporter family domain at 21–403 amino acid residues with an e-value of 3.3e-37. InterproScan server predicted Sodium/solute symporter domain (accession No. IPR032386) at 2–442 amino acid residues and Na/Glc_symporter_sf domain (accession No. IPR038377), at 2-448 amino acid residues. The solute is transported across the cytoplasmic membrane of prokaryotic and eukaryotic cells by sodium/substrate symport (or co-transport). Utilizing the energy stored in an electrochemical sodium gradient to drive the accumulation of solutes against a concentration gradient (sodium motive force, SMF).

Nature of subcellular localization

It is very important for predicting the subcellular localization of a hypothetical protein to further design a vaccine or drug target. It is also important to elucidate protein function. CELLO predicted subcellular localization analysis, which was confirmed by PSORTb, SOSUIGramN, and PSLpred. The HP's subcellular location was anticipated to be the inner membrane (Table 3). In contrast to THMM and HMMTOP, the inner membrane protein is highly predicted to contain transmembrane helices. The query protein was also predicted to be a transmembrane protein by the CCTOP server. All of these findings point to the protein being an inner membrane.

Table 3. Sub-cellular localization of hypothetical protein predicting from different servers

No.	Analysis	Result
1.	CELLO 2.5	InnerMembrane
2.	PSORTb	CytoplasmicMembrane
3.	SOSUIGramN	InnerMembrane
4.	PSLpred	InnerMembrane
5.	TMHMM 2.0	12 transmembrane helices present
6.	HMMTOP	25 transmembrane helices present
7.	CCTOP	Transmembrane protein

Secondary structure analysis

The secondary structures play important roles in protein structure and protein folding. All proteins functions are dependent on their structure, which, in turn, depends on physical and chemical parameters. In our study, the proportions of alpha-helix, beta-sheet content, coil

content, and overall confidence value were 79 %, 1 %, 32 percent, 20 %, and 85.2%, respectively, according to the PROTEUS Structure Prediction Server 2.0.

3D structure prediction, model quality refinement, and assessment

The three-dimensional structure of the target protein was predicted using the Raptorx server (http://raptorx.uchicago.edu/) and protein model 1 was chosen. The RaptorX program predicts 3D structures for protein sequences that have no close homologs in the Protein Data Bank (PDB) developed by the Xu group. A sequence input is used to predict secondary and tertiary structures, solvent accessibility, disordered regions, and solvent accessibility, according to RaptorX [41]. The Galaxy Refine server was used to refine the protein's projected tertiary structure, yielding five refined models and increasing the number of amino acid residues in the favored location. When compared to the other models, the scores listed above indicate the improved model's caliber. Tertiary model and refine model 1 were chosen and visualized in Discovery Studio (Figure 2 A and B). Through a Ramachandran plot analysis, PROCHECK evaluated the scalability of the galaxy server refined model, where the distribution of φ and ψ angles according to the model limits are depicted in Fig. 2C. A valid model covers 93.4% of the residues in the most preferred regions. A 3D structure model of the target sequence was validated by Verify3D and ERRAT and then compared against the established model. On the Verify3D graph, 91.77% of residues have an average 3D-1D score of ≥ 0.2, showing that the model has an excellent environmental profile, and the overall quality factor of 93.0804 in ERRAT indicates that the model is good. The YASARA energy minimization server later modified the 3D structure. Before energy minimization, the computed energy was –70,730.4 kJ/mol, but after energy minimization (by three rounds of steepest descent approach), it was reduced to –234,736.5 kJ/mol, making the modeled structure more stable. In addition, ProSA web server analysis resulted in a Z score of -7.01 which indicates the model validation (Figure 2D).

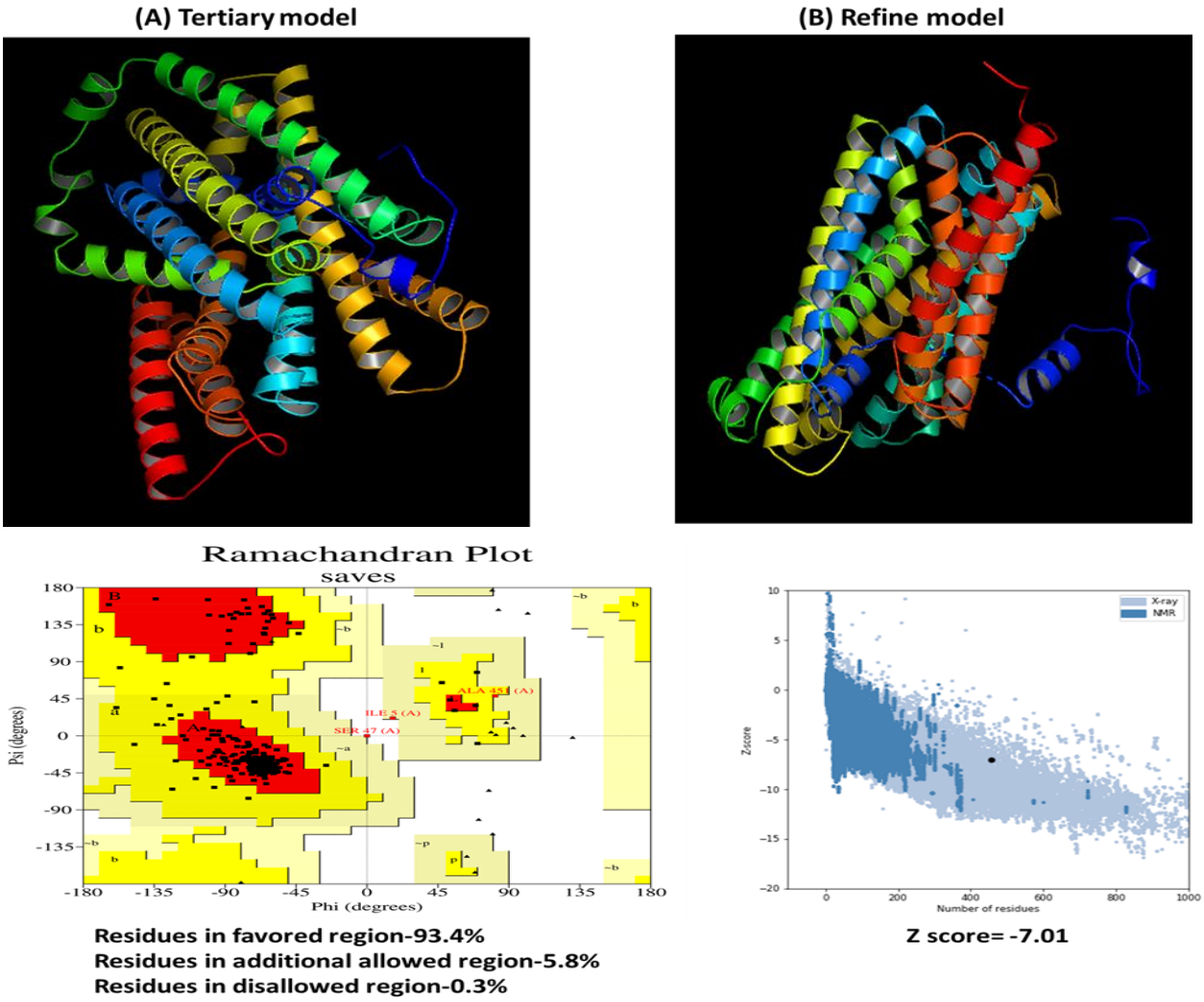


Fig 2 (A) Predicted tertiary structure of the hypothetical protein, (B) Refine model of the hypothetical protein from Galaxy refine server, (C) Ramachandran plot analysis of the refined model, and (D) Z-score results of the refined model from ProSA server.

Analysis of protein-protein interactions

We used the STRING 10.0 algorithm to make a prediction regarding the protein's possible functional interactions [31]. The identified functional partners with scores were; putA (0.905), GAC65625.1 (0.871), GAC65628.1(0.706), nanM (0.678), nanE (0.589), GAC63966.1 (0.554), nanA (0.524), GAC65383.1 (0.510) and acs (0.461). Of them, putA is a possible Proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase protein.

nanE and nanA is a Putative n-acetylmannosamine-6-phosphate 2-epimerase and N-acetylneuraminate lyase enzyme respectively (Fig 3).

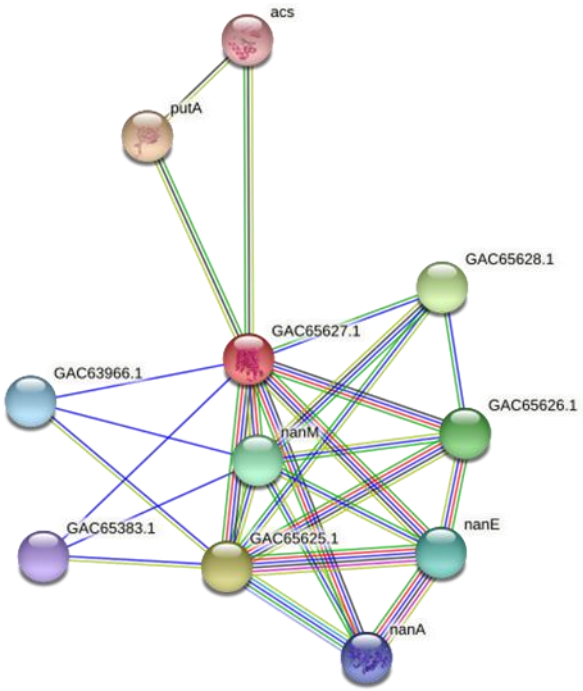


Fig 3. String (Protein-protein interactions) analysis of hypothetical protein

Table 4. CYSPRED and DIANA predict cysteine residues important in disulfide bonding

CYSPRED			**DIANA**	
Cysteine	**Prediction**	**Reliability**	**Distance**	**Bonded cysteine**
CYS 250	NON-Bonding State	9	184	AERQICQTQHY-VAQLSCAPVTA
CYS 404	NON-Bonding State	9		
CYS 435	NON-Bonding State	9		

Ligand binding interactions

Galaxy server ligand binding site predictions were done by matching target models with the PDB file of the best-predicted domain-A model. Three models were predicted by a galaxy server with different ligands. Galaxy server also combines the results into three parts Predicted ligand-binding residues, Predicted binding poses of the model, and Templates for protein-ligand complex (Table 5; Fig. 4 (A, B)). The details of the protein-ligand interaction

analysis were given in Table 5. The most probable protein-ligand binding poses and templates model for another protein-ligand complex was given in Fig 4.

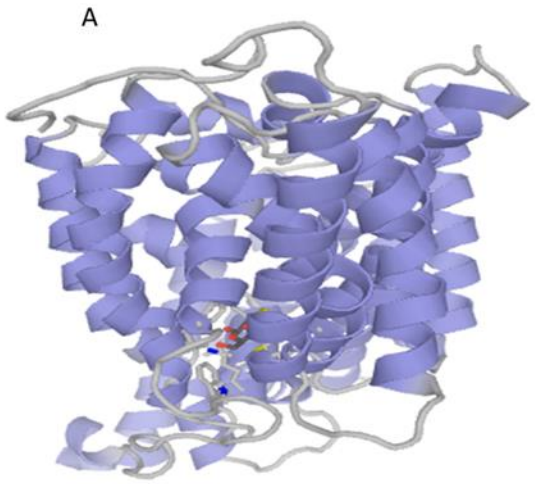
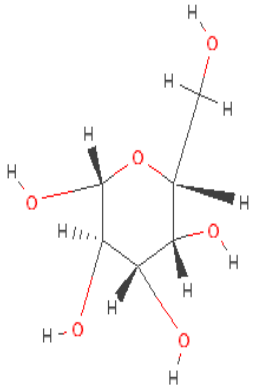
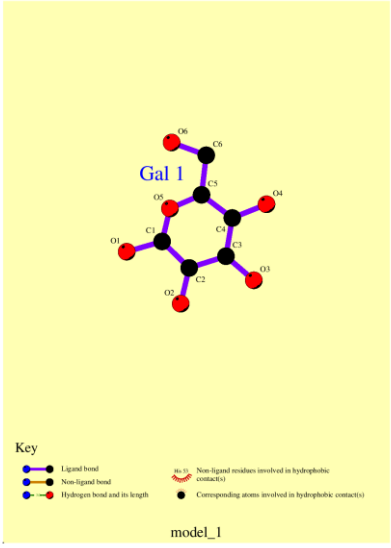
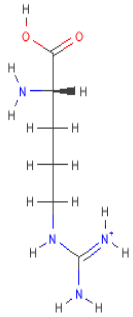
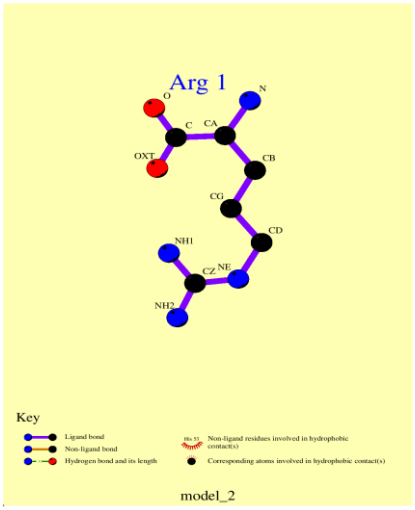


Fig. 4 (A) Predicted binding poses

Table 5. Predicted ligand-binding residues

No	Ligand Name	Ligand Structure	Binding residues	Interaction analysis
1	GAL		53K 65Y 210W 219M 364I 368M	
2	ARG		40T 42I 43G 44G 45G 122Q 168Q 169F 172I 304A 308S	

Active Site Detection

As predicted by the CASTp v.3.0 algorithm, the protein modeled contains 28 unique active sites (Fig 6). CASTp is a database server that can recognize regions on proteins, determine their boundaries, compute the area of the areas, and calculate the dimensions of the areas. Vacuums concealed within proteins and pockets on protein surfaces are also involved. To

define a pocket and volume spectrum or vacuum, surfaces of solvent-accessible molecules (Richard surface) and molecular surfaces (Connolly surface) are employed. CASTp might be utilized to look at the operational zones and surface properties of proteins. CASTp provides a dynamic, graphical user interface as well as on-the-fly measuring of user-submitted constructs [42]. Based on the area of 582.986 and the volume of 315.671, the top active sites of the model protein were identified (Fig 5). Fig 5 shows the protein's anticipated active site together with its amino acid residues.

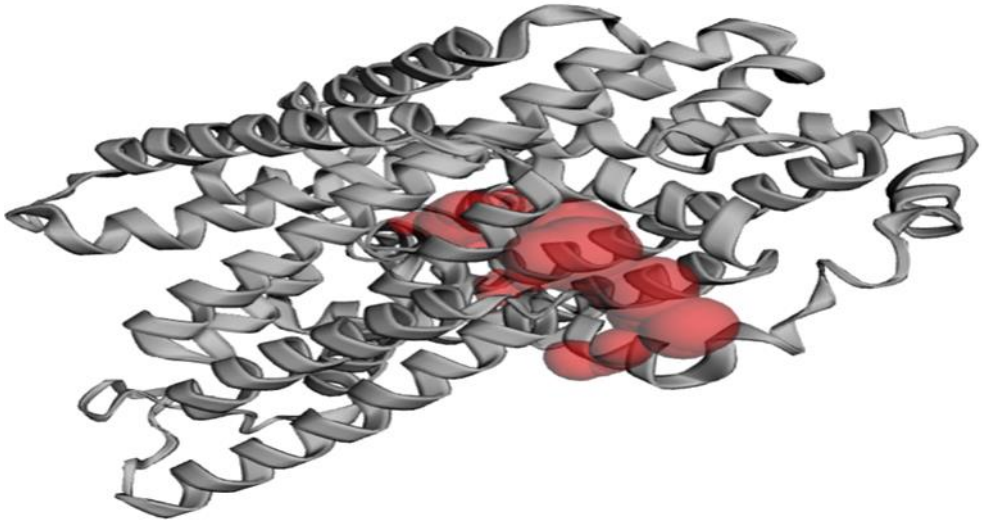


Fig 5. The hypothetical protein's active location. The red sphere represents the protein's active site

Fig 6. Active location of the hypothetical protein. The active site of amino acid residues (Blue color)

Molecular docking and simulation studies between hypothetical protein and chemotaxis protein

The docking prediction performed by the Cluspro server result showed 10 models for the docked complex. Model 1 (Figure 7 A) is considered to be the most probable model. After the selection of an appropriate model, a detailed investigation was done into the amino acid sequences that interact between the protein Chemotaxis and amino acid sequence of the hypothetical protein. The amino acid interactions revealed that the complex is stabilized by 2 hydrogen bonds (Fig 7B). Within the putA-hypothetical protein complex, Val1 and Thr91 of putA protein respectively interact with Gln192 and Leu292 of chemotaxis protein through H-bonds (Fig 7B). These H-bonds contribute towards the stability of the complex. The binding free energy of the protein complex was evaluated by the HawkDock server and found to be – 51.92 (kcal/mol) proving that the complex is stable. Additionally, three different binding sites of the protein complex was shown in Fig 7 (C, D, E).

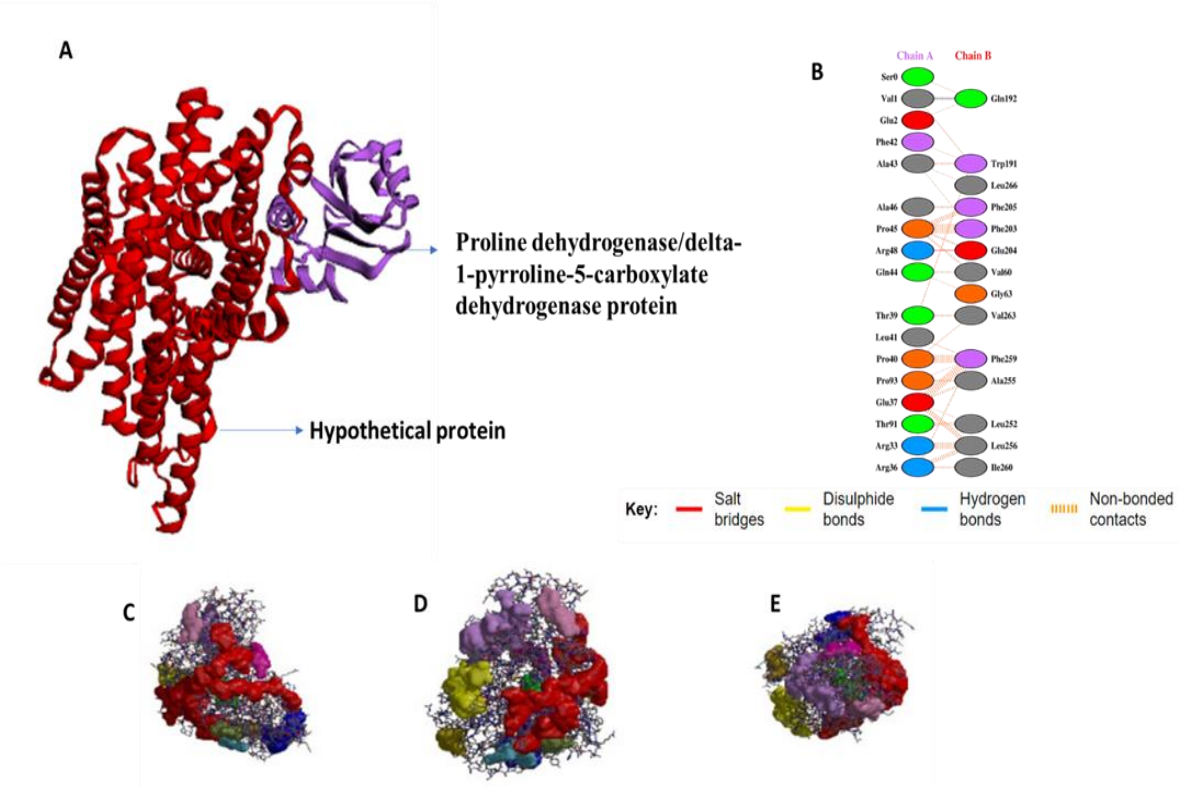


Fig. 7 (A) Chemotaxis-hypothetical protein complex, (B) Interactions between the protein-protein complex and (C, D, E) Three different binding sites of the selected protein-protein complex

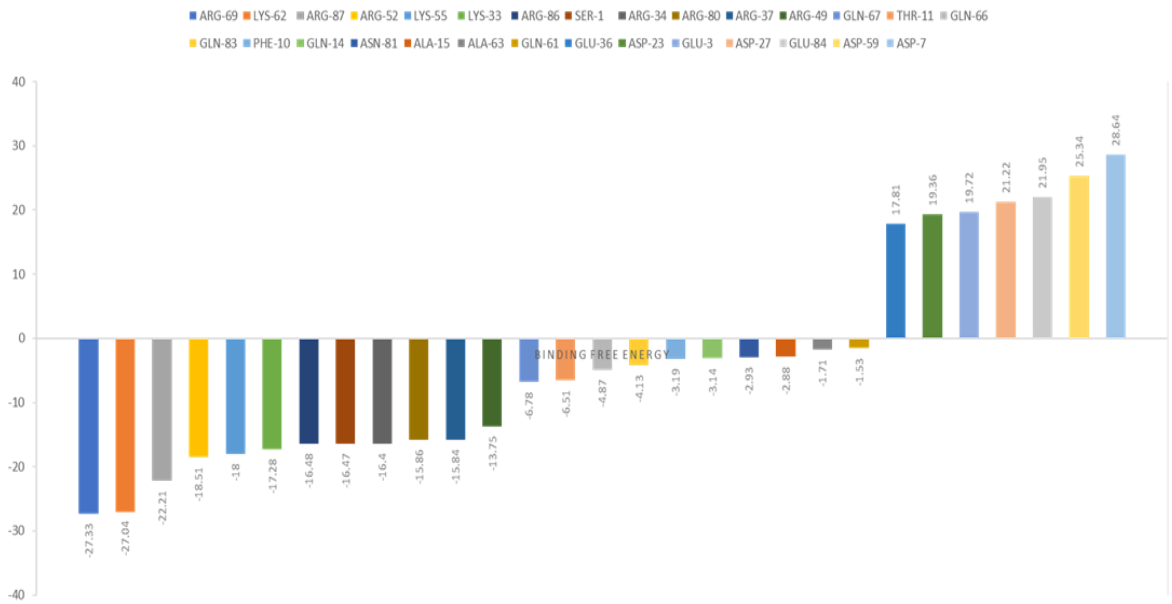


Fig 8 Bar graph of top 30 bindings free energy from HawkDock server

Conclusion

The hypothetical protein domain has a crucial role as a host cell invasion and apoptosis, according to the research. It was also discovered to be a soluble protein with a single exposed domain. The existence and distribution of this hypothetical protein domain across a wide range of bacterial strains and interactions with putA protein suggest that new antibacterial drugs could be developed. More research is being done, such as protein-ligand docking studies, to identify the representative amino acids involved in ligand binding. The molecular docking and simulation studies between hypothetical protein and putA protein are found in a stable interaction therefore it may be of interest to researchers looking to produce new drugs against multidrug resistance of *Edwardsiella tarda*.

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Research article

Yolci, M.S., R. Tunçtürk, and M. Tunçtürk, Farklı Ekstraksiyon Çözücüleri Kullanılarak Aspir (*Carthamus tinctorius* L.) Çiçeklerinin Toplam Fenol, Flavonoid Miktarları ve Antioksidan Aktivitesinin Belirlenmesi. International Journal of Life Sciences and Biotechnology, 2022. 5(1): p. 97-109.
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Farklı Ekstraksiyon Çözücüleri Kullanılarak Aspir (*Carthamus tinctorius* L.) Çiçeklerinin Toplam Fenol, Flavonoid Miktarları ve Antioksidan Aktivitesinin Belirlenmesi

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ÖZET

Bitkisel ürünlerden aktif bileşenlerin ekstraksiyon yöntemleriyle elde edilmesi, besin takviyeleri ve farmasötik ilaç endüstrisinin önemli süreçlerindendir. Bitki ekstraksiyonunda elde edilen ürünlerin kalitatif ve kantitatif sonuçları; çözücünün içeriği ve moleküler yapısı, çözünen maddenin içeriği ve hasat zamanı, ortam ısısı ve ekstraksiyonun süresi gibi birçok parametre ile ilişkilidir. Bu çalışmada farklı dönemlerde (çiçeklenme başlangıcından bir hafta sonra=HZ_1, çiçeklenme başlangıcından iki hafta sonra=HZ_2, çiçeklenme başlangıcından üç hafta sonra=HZ_3) hasat edilen aspir bitkisi çiçeklerinin farklı çözücüler (saf su, etanol, metanol ve aseton) kullanılarak ekstraksiyonları yapılmıştır. Üç farklı dönemde hasat edilen aspir çiçeklerine uygulanan farklı çözücülerin çiçeklerdeki toplam antioksidan aktivite (FRAP) ile toplam fenolik ve flavonoid madde miktarları değişimleri belirlenmiştir. Hasat zamanına göre en yüksek toplam fenolik madde miktarı (132.30 mg GA/100g) ile 2. hasat zamanından elde edilirken, en yüksek toplam flavonoid madde miktarı (19.15 mg QE/100g) ve toplam antioksidan aktivite (20.30 mg TE/g) 1. hasat zamanından tespit edilmiştir. Ayrıca, en yüksek toplam flavonoid madde miktarı (32.15 mg QE/100g) metanol ektraksiyonundan, en yüksek fenolik madde miktarı (224.05 mg GA/100g) ve toplam antioksidan aktivite miktarı (61.25 mg TE/g) ise saf su ektraksiyonundan belirlenmiştir. Aspir çiçeklerin çiçeklenme başlangıç dönemlerinde hasat edilmesi ve su ile ekstrakte edilmesi aspir çiçeklerinden maksimum düzeyde faydalanılabilirlik için önem arz etmektedir.

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ANAHTAR KELİMELER
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Determination of Total Phenol and Flavonoid Amounts and Antioxidant Activity of Safflower (*Carthamus tinctorius* L.) Flowers Using Different Extraction Solvents

ABSTRACT

Obtaining active components from herbal products by extraction methods is one of the significant processes of nutritional supplements and the pharmaceutical industry. Qualitative and quantitative results of the products derived from plant extraction; It is related to many parameters such as solvent content and molecular structure, solute content and harvest time, ambient temperature, and duration of extraction. In this study, safflower flowers harvested at different periods (one week after the beginning of flowering = HZ_1, two weeks after the beginning of flowering = HZ_2, three weeks after the beginning of flowering = HZ_3) were extracted using different solvents (pure water, ethanol, methanol, and acetone). The changes in the total antioxidant activity (FRAP) and the amount of total phenolic and flavonoid substances in the flowers of different solvents applied to the safflower flowers harvested in three different periods were determined. According to the harvest time, the highest total phenolic substance content (132.30 mg GA/100g) was derived from the 2nd harvest time, while the highest total flavonoid substance amount (19.15 mg QE/100g) and total antioxidant activity (20.30 mg TE/g) were determined from 1. harvest time. In addition, the highest amount of total flavonoid substance (32.15 mg QE/100g) was obtained from methanol extraction, the highest amount of phenolic substance (224.05 mg GA/100g), and total antioxidant activity amount (61.25 mg TE/g) were determined from pure water extraction. Harvesting safflower flowers at the beginning of flowering and extracting them with water is important for maximum utilization of safflower flowers.

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Giriş

Fenolik maddeler; protein, yağ, vitamin ve karbohidrat gibi primer metabolitlerden farklı yollarla oluşturulmaktadır. Fenolik bileşikler, fenolik asitler ve flavonoidler olarak iki ana gruba ayrılmaktadırlar. Enzimatik ve enzimatik olmayan birçok çeşidi ile canlılarda antioksidan aktivite göstermektedirler. Bitkilerde fenolik bileşikler; büyüme-gelişme, tozlaşma, üreme, pigment oluşturma, serbest radikalleri nötralize etme, çeşitli patojen ve olumsuz çevre şartlarında bitkiyi korumaya alma ve direncini arttırma gibi birçok farkı amaçlarla üretilmektedir [1].

İnsan bedeninde doğal olarak gerçekleşen metabolik faaliyetler ve çeşitli çevresel kirleticiler sebebiyle serbest radikaller oluşmakta ve birçok organ ve sistemde tahribat ve hastalıklara neden olduğu bildirilmektedir [2]. Tüm canlılarda olduğu gibi insan vücudunda da varolan antioksidan sistemler sayesinde serbest radikallerin verdiği zararlar önlenmeye çalışılmakta ancak yetersiz kalındığı durumların da olduğu görülmektedir. Bitkisel kaynaklardan alınan antioksidan yapıdaki fenolik bileşikler sayesinde oksidan-antioksidan dengenin sağlandığı bilinmektedir [3]. Sentetik

antioksidan tüketimine bağlı olarak olumsuz bazı etkilerle karşılaşılması nedeniyle doğal kaynaklı antioksidanlar insan sağlığı için daha etkili ve sağlıklı olduğu ve daha fazla talep gördüğü bildirilmektedir [29].

Aspir veya yalancı safran olarak bilinen *Carthamus tinctorius* L. Compositae/Asteraceae familyasına mensup tek yıllık bir bitkidir. Güney Asya, Hindistan, İran, Çin ve Mısır gibi kurak iklimlerde doğal olarak yetişmekte olan aspir bitkisinin birçok ülkede kültürü yapılmaktadır [4]. Aspir bitkisinin vejetatif kısımları, tohum ve çiçekleri dünyanın birçok ülkesinde beslenme, tedavi, biyogaz, yem, süs, gıda ve tekstil ürünlerinde renk verici olarak kullanılmaktadır [5]. Aspir bitkisinin tohumları % 70 oranında linoleik asit, % 10 civarında oleik asit ve az miktarda stearik asit içerdiği ve bu nedenle ilk zamanlardan beri yemeklik yağ ve kuş yemi olarak tüketildiği belirtilmektedir [6].

Aspir taç yaprakları gallik asit, klorojenik asit, siringik asit, kuarsetin ve epikateşin gibi fenolik bileşikleri ve flavonoid yapıdaki glikozil-qinokalkonlar olarak bilinen renk maddelerini barındırdığı bilinmektedir. [7]. HSYA (Hydroxy Safflor Yellow A), aspir çiçeğinin renk pigmentlerinin büyük bir kısmını oluşturmaktadır. Aspir çiçeklerinin terapötik etkilerinin büyük çoğunluğunu bu madde belirlediği için özellikle Çin farmakopesinde yer almaktadır. Aspir çiçekleri içerdiği zengin fenolik, antioksidan ve diğer maddelerden ötürü kalp damar koruyucu, karaciğer temizleyici, antikanser, metabolizma düzenleyici, akciğer ve sinir sistemi koruyucu gibi birçok hastalığın tedavisinde geniş kullanım alanı bulmuştur [8]. Aspir bitkisinde bulunan fenolik yapıdaki renk pigmentlerinin; sulama, gübreleme, ekim sıklığı, ekim zamanı, hasat zamanı, çeşit, çevresel stres durumları gibi birçok parametreden etkilendiği ve miktarca değişkenlik gösterebildiği çalışmalarla tespit edilmiştir [9, 10, 11]. Bitkilerden hasat sonrası elde edilen çiçeklerin sekonder metabolitlerinin; sıcaklık, UV ışınları, pH, çeşitli gazlar, metal iyonları ve kimyasallar gibi farklı etkenlere bağlı olarak değişime uğradığı bildirilmiştir [12]. Aspirde birer hafta ara ile çiçek hasadının flavanoid yapıdaki kartamidin maddesi üzerine etkisinin araştırıldığı çalışmada, çiçeklenme başlangıcı (1. hafta) ile üçüncü haftaya kadar yapılan hasatlarda kartamidin miktarının gittikçe arttığı, üçüncü haftadan sonraki hasatlarda ise gittikçe düşüşler gözlendiği bildirilmiştir [13]. Aspir çiçeklerinde farklı çözücülerin (aseton, metanol, etanol) ve hasat zamanlarının

fenolik madde ve antioksidan aktivite üzerine etkilerinin araştırıldığı çalışmada aseton/su (2/8) ekstraksiyonunun en yüksek polifenol ve antioksidan aktivite gösterdiği, hasat zamanlarının antioksidan içeriklerinde farklılıklar oluşturduğu ve en yüksek antioksidan aktivitenin tam çiçeklenme zamanında, fenolik madde miktarında ise tam açılma döneminde elde edildiği bildirilmişti [7].

Bu çalışma, farklı zamanlarda hasat edilen aspir çiçeklerinin farklı çözücülerle ekstraksiyonunun yapılarak toplam fenolik madde, toplam flavonoid madde ve antioksidan aktivitenin belirlenmesi amacıyla yürütülmüştür.

Materyal ve Metot

Deneme materyali olarak kullanılan Asol aspir çeşidinin çiçekleri Van YYÜ Ziraat Fakültesi Tarla Bitkileri Uygulama Alanı'nda 2020 yılı sulu şartlar altında yetiştirilen aspir bitkisinden farklı dönemlerde (çiçeklenme başlangıcından bir hafta sonra=HZ_1, çiçeklenme başlangıcından iki hafta sonra=HZ_2, çiçeklenme başlangıcından üç hafta sonra=HZ_3) hasat edilmiştir. Çiçek tablalarından ayrılan petaller oda sıcaklığında kurutulup daha sonra paketlenmiştir. Çiçek ektraksiyonunda çözücü olarak saf su=$EÇ_1$, etanol=$EÇ_2$ (%99.5/Merck), metanol=$EÇ_3$ (%99.7/Merck) ve aseton=$EÇ_4$ (%99.5/Merck) kullanılmıştır. Çiçek örneklerinden 2'şer gr alınıp falkon tüplerine konulmuştur. Daha sonra üzerine çözücülerden 4'er ml eklenmiş ve homojenizatörde ekstrakte edilmiştir. Örnekler santrifüjden geçirilmiş ve süpernatant kısmı ayrılıp analizlere hazır hale getirilmiştir. Aspir çiçeklerinin hasat edildiği döneme ait iklim verileri Tablo 1'de, toprak verileri ise Tablo 2'de verilmiştir.

Toplam fenolik madde miktarı (mg GA/100g)

Toplam fenolik madde miktarının belirlenmesinde; Obanda ve ark., (1997) tarafından belirtilen Folin-Cicaltea spektrofotometrik yöntemin modifiye edilmesiyle geliştirilen yöntem kullanılmıştır [14]. Folin-Cicaltea çözeltisi 1:3 oranında seyreltilmiştir. Doygun sodyum karbonat (%35) çözeltisi; 87.5 g sodyum karbonat distile suda çözdürülüp 250 ml'ye tamamlanarak bir gece bekletilmesinin ardından filtre edilmiştir. Gallik asit (GA) stok çözeltisi (500 µg/ml); 100 ml saf suda 50 mg gallik asit çözdürülerek hazırlanmıştır. Gallik asit çalışma çözeltisi; 500 µg/ml gallik stok çözeltisinden her biri 5'er ml'lik ölçü balonlarında, konsantrasyonu 0-55 µg/ml arasında değişen 9 ayrı çözelti olarak hazırlanmıştır. Bu çözeltilerden 1 ml alınarak 1 ml Folin-

Cicaltea çözeltisi ile karıştırılmıştır. 5 dk bekletildikten sonra 2 ml sodyum karbonat ilave edilerek çalkalanmış ve 2 ml su ile seyreltilmiştir. Bu karışım 60 dk karanlık ortamda bekletildikten sonra spektrofotometrede 700 nm dalga boyunda absorbans değeri okunmuştur. Gallik asidin bu farklı konsantrasyonlarına karşı okunan absorbans değerlerinin grafiğe dönüştürülmesi ile kalibrasyon eğrisi elde edilmiştir (r^2= 0.987).

Tablo 1 Van ili tuşba ilçesi 2020 yılı meteorolojik verileri

Table 1 Meteorological data for the year 2020 of Tusba district of Van province

Aylar	**Ortalama Sıcaklık (ºC)**	**Toplam Yağış (mm)**	**Ortalama Nem (%)**
Ocak	-2,5	43,8	74,5
Şubat	-1,7	79,9	77,1
Mart	4,9	40,9	72,5
Nisan	8,6	50,9	65,4
Mayıs	14,5	27,8	54
Haziran	19,3	13,4	44,4
Temmuz	23	17,9	46,4
Ağustos	21,6	10	44,5
Eylül	20,1	5,6	41,3
Ekim	13,3	1,8	47,2
Kasım	6,7	12,8	65,5
Aralık	1,4	27,7	71,4
Ortalama	10,76	27,7	58,68

Toplam flavonoid madde miktarı (mg QE/100g)

Toplam flavonoid madde miktarının belirlenmesi; Toplam flavonoid madde tayini Quettier-Deleu (2000)'nın geliştirmiş oldukları yönteme göre belirlenmiştir [15]. 2 ml ekstrakt üzerine 2 ml %2'lik $AlCl_3$ eklenerek oda sıcaklığında ve karanlıkta 1 saat bekletilmiştir. Her örnekte 2 paralel çalışma yapılarak ekstrelerin toplam flavonoid içerikleri, 415 nm dalga boyunda spektrofotometre ile ölçülmüş ve standart quarsetin (QE) kullanılarak hazırlanmış olan kalibrasyon eğrisinden yararlanılarak mg QE/100 g cinsinden hesaplanmıştır (r^2= 0.998).

Toplam antioksidan aktivite miktarı (mg TE/g)

Toplam antioksidan aktivitesinin belirlenmesi (FRAP) (mg Trolox Eşdeğeri (TE)/g); aspir çiçeklerinden 2 g tartılıp üzerine 4 ml farklı çözücüler ayrı ayrı (metanol, etanol, aseton ve saf su) eklenerek homojenizatörden geçirilen materyal 10 dk 10000 rpm'de

santrifüj edildikten sonra üstte kalan süpernatant kısmı alınmıştır. Daha sonra 300 mM asetat tamponu (pH 3.6), 40 mM HCl'de çözülerek hazırlanan 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ), 20 mmol/L $FeCl_3.6H_2O$ çözeltileri hazırlandıktan sonra sırası ile 10:1:1 oranında karıştırılıp FRAP ayıracı hazırlanmıştır. Aspir çiçeklerine 2850 µL FRAP ayıracı ile ABTS (2,2-Azinobis 3-ethyl-benzothiazoline-6-sulfonic acid) analizi için hazırlanan karışım ayrı ayrı çözücüler ile 50 kat seyreltildikten sonra alınan 150 µL örnek karıştırılıp oda sıcaklığında 60 dk bekletilmiştir. Oluşan ferrustripiridiltriazin kompleksi spektrofotometrede 593 nm'de ölçülmüş ve sonuçlar mg Trolox/g olarak belirtilmiştir [16]. Trolox konsantrasyon aralığı 0-500 ppm olarak çalışılmıştır (r^2= 0.995).

Tablo 2 çiçek örneklerinin alındığı toprağa ait bazı fiziksel ve kimyasal özellikler

Table 2 Some physical and chemical properties of the soil from which flower samples were taken

Derinlik (cm)	Tekstür	pH	Kireç (%)	Toplam Tuz (µS/cm)	Organik Madde (%)
0-20	Kumlu-Tınlı	7,65	188	8,8	0,94
20-40	Kumlu-Tınlı	7,73	152,1	9,1	0,63

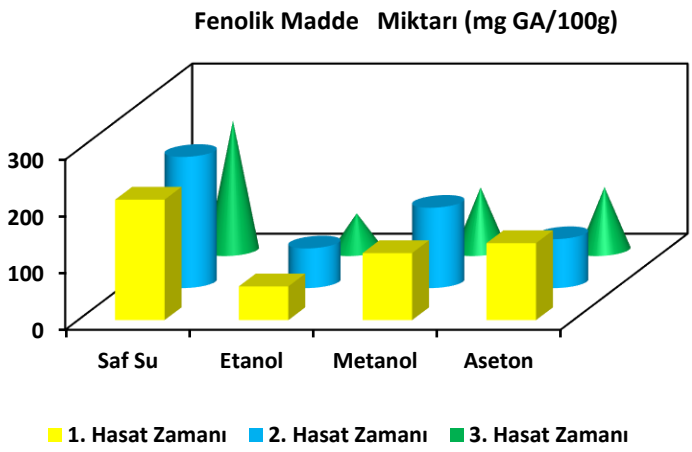
İstatistiksel veriler

Elde edilen verilerin istatistiksel analizleri COSTAT (sürüm 6.03) paket programı ile çoklu karşılaştırma testleri ise LSD testine göre yapılmıştır.

Bulgular ve Tartışma

Çalışma bulgularına göre hasat zamanı, çözücü ve hasat zamanı × ekstraksiyon çözücüsü interaksiyonun toplam fenolik madde miktarı üzerine etkisi istatistiki olarak %5 oranında önemli görülmüştür. Hasat zamanı bulgularına göre en yüksek değer 132.30 mg GA/100g ile 2. hasat zamanından, en düşük değer ise 128.46 mg GA/100g ile 3. hasat zamanından elde edilen çiçek örneklerinde tespit edilmiştir. Çözücü uygulamalarına göre en yüksek değer 224.05 mg GA/100g ile saf su ekstraksiyonundan, en düşük değer ise 64.59 mg GA/100g ile etanol ile ekstrakte edilen örneklerden elde edilmiştir. Hasat zamanı × ekstraksiyon çözücüsü interaksiyonunda en yüksek değer 231.32 GA/100g ile HZ_2 × $EÇ_1$ interaksiyonundan elde edilmiştir. HZ_3 × $EÇ_1$ interaksiyonu ile istatistiksel olarak fark görülmemiştir (Tablo 3, Şekil 1). Ayçiçeğinde

farklı hasat zamanlarının toplam fenolik madde ve antioksidan aktivite üzerine önemli etkilerinin olduğunu, toplam fenolik madde miktarı, tomurcuklanma-çiçeklenme başı-tam çiçeklenme sürecinde gittikçe arttığını ancak tam çiçeklenmeden sonraki hasatlarda azaldığını ayrıca toplam antioksidan aktivitenin tam çiçeklenmede en yüksek, çiçeklenme başlangıcında ise en düşük değeri aldığını bildirmişlerdir [17]. Farklı bitkilerle yapılan çalışmalarda hasat zamanının uzamasına bağlı olarak toplam fenol, flavonoid ve antioksidan aktivite değerlerinde düşüşlerin gözlendiği bildirilmiştir [18, 19, 20, 21, 22].

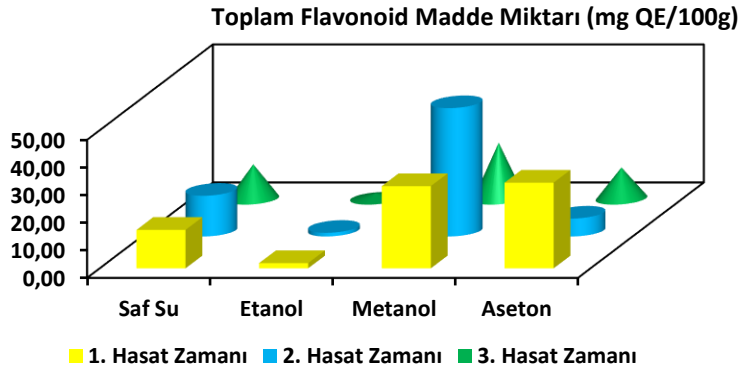


Şekil 1 Farklı hasat zamanı ve ekstraksiyon çözücülerinin aspir çiçeklerinin toplam fenolik madde miktarı üzerine etkisi

Fig 1 The effect of different harvesting times and extraction solvents on the total phenolic content of safflower flowers

Hasat zamanı, ekstraksiyon çözücüsü ve hasat zamanı × ekstraksiyon çözücüsü interaksiyonunun toplam flavonoid madde miktarı üzerine istatistiksel olarak %5 oranında önemli etkisi görülmüştür. Hasat zamanı verilerine göre en yüksek değer 19.15 mg QE/100g ile 1. hasat zamanından, en düşük değer ise 11.40 QE/100g ile 3. hasat zamanından elde edilen çiçek örneklerinde tespit edilmiştir. Çözücü uygulamalarına göre en yüksek değer 32.15 mg QE/100g ile metanol ekstraksiyonundan, en düşük değer ise 1.69 mg QE/100g ile etanol ekstraksiyonundan elde edilmiştir. Hasat zamanı × ekstraksiyon çözücüsü interaksiyonunda en yüksek değer 46.49 QE/100g ile 2. Hasat zamanında elde edilen çiçeklerin metanol ile ekstraksiyonundan tespit edilmiştir. (Tablo 3, Şekil 2). *Vaccinium vitisidaea* L. bitkisinin farklı organlarından (kök, yaprak, meyve) farklı zamanlarda yaptıkları hasatlarda yaprak örneklerinde, hasat zamanının uzamasına

bağlı olarak toplam fenol ve toplam antioksidan aktivitede azalmaların olduğu ancak diğer organlarda aynı durumun olmadığını belirtmişlerdir [23]. *Lycopus lucidus* bitkisinde hasat zamanlarının gecikmesine bağlı olarak toplam fenolik madde ve antioksidan aktivitenin azaldığı, fenolik içerikler açısından ise bazı komponentlerin artarken bazılarının ise azaldığı tespit edilmiştir [24].

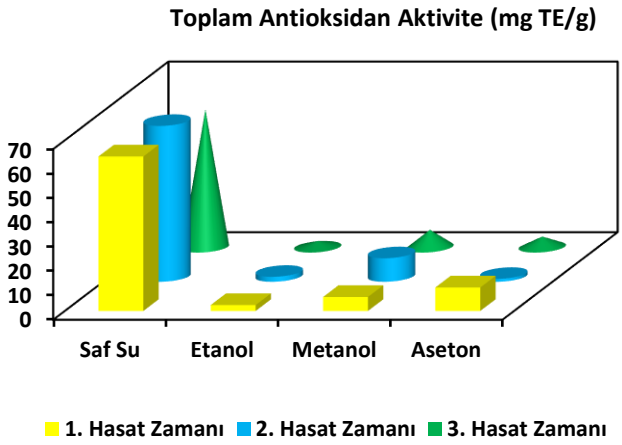


Şekil 2 Farklı hasat zamanı ve ekstraksiyon çözücülerinin aspir çiçeklerinin toplam flavonoid madde miktarı üzerine etkisi

Fig 2 The effect of different harvesting times and extraction solvents on the total flavonoid content of safflower flowers

Toplam antioksidan aktivite miktarı üzerine hasat zamanı, ekstraksiyon çözücüsü ve hasat zamanı × ekstraksiyon çözücüsü interaksiyonunun etkisi istatistiki olarak %5 düzeyinde önemli olduğu belirlenmiştir. Hasat zamanı verilerine göre, en yüksek toplam antioksidan aktivite 20.30 mg TE/g ile 1. hasat zamanından, en düşük değer ise 17.51 mg TE/g ile 3. hasat zamanında elde edilen çiçeklerde görülmüştür. Çözücü uygulamalarına göre en yüksek değer 61.25 mg TE/g ile su ekstraksiyonundan, en düşük değer ise 2.28 mg TE/g ile etanol ektraksiyonundan tespit edilmiştir. Hasat zamanı × ekstraksiyon çözücüsü interaksiyonunda en yüksek değer 63.94 mg TE/g ile 2. hasat zamanı aspir çiçeklerinin su ile ektrakte edilen örneklerinden elde edilmiş ve $HZ_1 \times EÇ_1$, $HZ_3 \times EÇ_1$ interaksiyonları ile istatistiki olarak farklılık göstermemiştir (Tablo 3, Şekil 3). Bazı araştırıcılar ise yaptıkları çalışmalarında hasat zamanın uzamasına bağlı olarak toplam fenol, flavonoid ve antioksidan aktivitenin değişmediğini, dalgalı sonuçlar verdiğini tespit etmişlerdir [25, 26]. Literatürler incelendiğinde, bitkilerde toplam fenolik, flavonoid ve antioksidan aktivite miktarlarının hasat zamanlarına göre değişime uğradıklarıanlaşılmaktadır. Bu farklılıkların ana nedenlerinin; kullanılan çeşit veya popülasyonların genetik farklılığı, lokasyon, çevresel stres durumları, bitkinin

gelişim aşaması, hasat saatleri ve hangi organdan örnek alındığı gibi etkenlerolduğu sonucuna varılmış ve önceki çalışma verilerinin sonuçlarımızla uyum gösterdiği tespit edilmiştir.



Şekil 3 Farklı hasat zamanı ve ekstraksiyon çözücülerinin aspir çiçeklerinin toplam antioksidan aktivite üzerine etkisi

Fig 3 Effect of different harvesting times and extraction solvents on total antioxidant activity of safflower flowers

Farklı bitkilerle yapılan çalışmalarda toplam fenol, flavonoid ve antioksidan aktivite değerlerinin çözücülere bağlı olarak değiştiği her bitki ve elde edilecek içerik için farklı çözücü kullanılması gerektiği belirtilmiştir [7, 27, 28]. Beslenme veya tedavi amaçlı kullanılan bitkilerden yüksek seviyede fayda sağlanabilmesi için ilk aşamanın fitokimyasalların en uygun ektraksiyon yöntemleriyle elde edilmesi gerektiği ortaya konulmuştur [29].

Fenolik bileşiklerin ekstraksiyonunda çözücü kimyasalların yapı ve partikül boyutu, ekstraksiyonun metodu, ortamda bulunan ve fenolik bileşiklerle etkileşime girebilecek özellikteki maddelerden (proteinler, karbonhidratlar vb.) etkilenmektedir.

Tablo 3 Farklı hasat zamanı ve ekstraksiyon çözücülerinin aspir çiçeklerinin bazı biyokimyasal parametreler üzerine etkisi

Table 3 The effects of different harvesting times and extraction solvents on some biochemical parameters of safflower flowers

UYGULAMALAR		Toplam Fenolik Madde Miktarı (mg GA/100g)	Toplam Flavonoid Madde Miktarı (mg QE/100g)	Toplam Antioksidan Aktivite (mg TE/g)
Hasat Zamanı (HZ)	**Ekstraksiyon Çözücüsü (EÇ)**			
HZ_1	**$EÇ_1$**	212.39 b	13.92 e	63.39 a
	$EÇ_2$	59.54 h	1.89 i	2.44 cd
	$EÇ_3$	118.29 d	29.78 c	5.75 bc
	$EÇ_4$	135.79 c	31.03 b	9.64 b
ort		**131.5 AB**	**19.15 A**	**20.30 A**
HZ_2	**$EÇ_1$**	231.32 a	14.75 e	63.94 a
	$EÇ_2$	69.89 g	1.32 i	2.03 cd
	$EÇ_3$	141.68 c	46.49 a	9.64 b
	$EÇ_4$	86.32 f	6.49 h	1.18 d
ort		**132.30 A**	**17.26 B**	**19.19 AB**
HZ_3	**$EÇ_1$**	228.46 a	12.36 f	56.44 a
	$EÇ_2$	64.36 gh	1.89 i	2.38 cd
	$EÇ_3$	110.25 e	20.18 d	7.00 bc
	$EÇ_4$	110.79 e	11.20 g	4.22 cd
ort		**128.46 B**	**11.40 C**	**17.51 B**
Ekstraksiyon Çözücüsü	**$EÇ_1$**	*224.05 A*	*13.67 C*	*61.25 A*
	$EÇ_2$	*64.59 D*	*1.69 D*	*2.28 C*
	$EÇ_3$	*123.40 B*	*32.15 A*	*7.46 B*
	$EÇ_4$	*110.96 C*	*16.24 B*	*5.01 BC*
HZ (LSD %5)		3.75 *	0.38 *	2.39 *
EÇ (LSD %5)		4.33 *	0.44 *	2.76 *
HZ × EÇ (LSD %5)		13.01*	1.34 *	8.28*
VK (Varyasyon Katsayısı)		3.41	2.88	14.93

* LSD testine göre %5 önemli. Aynı sütunda aynı küçük harfle gösterilen ortalamalar arasında %5 seviyesinde herhangi bir fark yoktur. Aynı sütunda aynı büyük koyu harfle gösterilen ortalamalar arasında %5 seviyesinde herhangi bir fark yoktur. Aynı sütunda aynı italik büyük harfle gösterilen ortalamalar arasında %5 seviyesinde herhangi bir fark yoktur.

Fenoliklerin çözünürlüğü kullanılan solvent türü, fenoliklerin polimerizasyon derecesi ve diğer bileşenlerle çözünmez nitelikte kompleksler oluşturması, ekstraksiyon süresi ve sıcaklığı, örnek:solvent oranı gibi faktörlere bağlı olarak değişmektedir. Bu nedenle bitkisel materyallerden fenolik bileşiklerin ekstraksiyonu için tamamen uygun bir ekstraksiyon prosedürü bulunmamaktadır. Metanol, etanol, aseton, su, etilasetat ve

bunların uygun oranlarda kombinasyonları fenolik bileşen ekstraksiyonunda yaygın olarak kullanılmaktadır [27].

Sonuç

Çalışma sonucunda hasat zamanına göre toplam flavonoid madde miktarı ve toplam antioksidan aktivite değerleri en yüksekten en aza doğru $HZ_1 > HZ_2 > HZ_3$ şeklinde sıralanırken, toplam fenolik madde miktarları ise $HZ_2 > HZ_1 > HZ_3$ şeklinde sıralanmıştır. Çözücülere göre toplam fenolik madde miktarı ve toplam antioksidan aktivite değerleri saf su > metanol > aseton > etanol şeklinde sıralanırken, toplam flavonoid madde miktarı değerleri ise metanol > aseton > saf su > etanol şeklinde sıralanmıştır. En yüksek toplam flavonoid madde miktarı ve antioksidan aktivite için en uygun hasat zamanının HZ_1 olduğu, toplam fenolik madde miktarı için ise HZ_2 olduğu tespit edilmiştir. Çözücüler göz önüne alındığında en yüksek toplam fenolik madde miktarı ve toplam antioksidan aktivite için en uygun çözücünün saf su olduğu, toplam flavonoid madde miktarı için ise metanol olduğu tespit edilmiştir. Sonraki çalışmalarda farklı çözücülerin denenmesi ve çalışılan çözücülerin birbirleriyle farklı oranlarda karıştırılarak ekstraksiyonda kullanılması tavsiye edilmektedir.

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Derleme makale / Review article

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İnsan Endojen Retrovirüslerin Kanserle Olan İlişkisinin İncelenmesi

Abdullah Karaman[1] , Elif Karlık[2*]

ÖZET

Transpozonlar, genomdaki yerlerini değiştirebilme özelliğine sahip olan hareketli DNA parçalarıdır. Transpozonlar genomdaki yer değiştirme işlemini, transpozisyon olarak adlandırılan bir mekanizma ile gerçekleştirmekte ve sahip oldukları transpozisyon mekanizmasına göre DNA ve RNA transpozonları olarak iki alt sınıfa ayrılmaktadırlar. Retrotranspozonlar olarak da adlandırılan RNA transpozonları, insanın evrim sürecinde önemli rol alan endojen retrovirüsleri (ERV) içermektedir. İnsan genomunun yaklaşık %8'ini oluşturan insan endojen retrovirüsleri (HERV) 3 sınıf altında toplanmadır. İkinci sınıfta yer alan insan endojen retrovirüs K (HERV-K), insan genomuna yakın sayılabilecek bir zamanda entegre olan, insan genomundaki en aktif HERV'dir. Ovaryum, meme ve deri kanseri gibi çeşitli kanser türlerinin ortaya çıkmasında HERV-K'nın rol aldığı görülmektedir. HERV'lerin kanser gelişimi ile olan ilişkisi uzun süredir araştırılmaktadır. Kanser hücrelerinde HERV proteinleri saptanmış olsa da HERV'lerin kanser gelişimindeki rolü kesin olarak anlaşılamamıştır. Son dönemde yapılan çalışmalar kanser hücrelerinde yüksek seviyede anlatım yaptığı gösterilen HERV proteinlerinin, kanser tedavisinde rol alan immün yanıt için ana hedef olarak kullanılabileceğini ortaya koymaktadır. Histon deasetilaz inhibitörleri ve kontrol noktası inhibitörlerinin kombinasyonundan oluşan yeni yaklaşımlar da kanser tedavisinde kullanılmak üzere test edilmektedir. HERV anlatımı, interferon tip 1 yanıtını etkinleştiren, sitozoldeki tek iplikli RNA'nın kalıp tanıma reseptörlerini aktive ederek immün sistem yanıtını başlatmaktadır. Bunun sonucunda CD8 T hücreleri tarafından gerçekleştirilen kanser hücresi tanınması arttırılarak kanser gelişiminin engellenebileceği öngörülmektedir. Histon deasetilaz ve kontrol noktası inhibitörlerinin kombinasyonundan meydana gelen bu yeni yaklaşım, anti-tümör aktivitesini arttırarak kanser tedavisinde yeni bir umut oluşmasına olanak sağlayacaktır. Bu derlemede, HERV'lerin kanser oluşumundaki etkilerini ortaya koyan çalışmalar özetlenerek HERV'ler ile ilişkili yeni tedavi yaklaşımları incelenecektir.

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ANAHTAR KELİMELER
Transpozonlar,
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Investigation of the Relationship of Human Endogenous Retroviruses with Cancer

ABSTRACT

Transposons are mobile elements of DNA that have the ability to change their locations in the genome. Transposons perform the displacement process in the genome by a mechanism called transposition and are divided into two subclasses as DNA and RNA transposons according to their transposition mechanism. RNA transposons also called retrotransposons including endogenous retroviruses (ERVs) that play an important role in human evolution. Human endogenous retroviruses (HERV) constituting about 8% of the human genome are grouped under 3 classes. Human endogenous retrovirus K (HERV-K) in the second class is the most active HERV in the human genome considered to integrate in human genome in a close time. It is observed that HERV-K plays a role in the emergence of various cancer types such as ovarian, breast and skin cancer. The relationship of HERVs to cancer development has been investigated for a long time. Although HERV proteins were detected in cancer cells, the role of HERVs in cancer development has not been clearly understood. Recent studies revealed that HERV proteins in high levels in cancer cells can be used as the main target for the immune response involved in cancer therapy. New approaches in combination of histone deacetylase inhibitors and checkpoint inhibitors are also being tested for use in cancer therapy. HERV expression initiates the immune system response by activating the pattern recognition receptors of single-stranded RNA in the cytosol, activating the interferon type 1 response. As a result, it is predicted that cancer development can be prevented by increasing the recognition of cancer cells by CD8 T cells. This new approach consisting of a combination of histone deacetylase and checkpoint inhibitors will increase its anti-tumor activity and provide new hope in cancer therapy. In this review, we will summarize recent studies revealing the effects of HERVs on cancer formation, and new treatment approaches related to HERVs will be examined.

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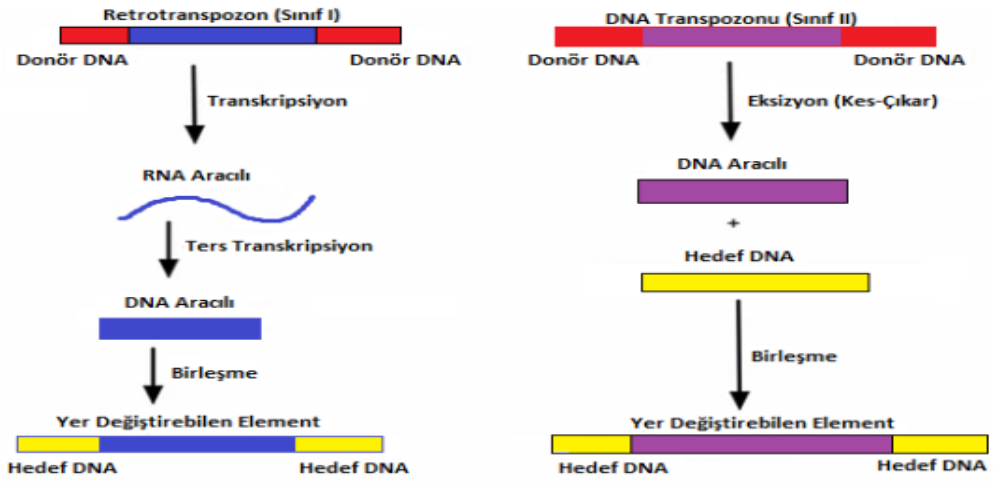
Cancer

Giriş

Bir organizmanın genetik materyalinin tamamı genom olarak adlandırılmaktadır [1]. İnsan genomundaki genlerin yaklaşık %1-1.5'lik kısmının kodlama yaptığı; %98,5'lik kısmının ise kodlama yapmayan genlerden (non-coding DNA) ve gen dışı bölgelerden oluştuğu bilinmektedir. Herhangi bir protein kodlamasına katılmayan bu genler, belirli bir zamana kadar bilim insanları tarafından 'junk DNA' olarak adlandırılmışlardır [2]. Ancak son yıllarda yapılan çalışmalarla, bu genlerin direkt olarak protein kodlamasalar da; proteinlerin hangi dokuda ve ne miktarda kodlanacağını düzenlemek gibi önemli görevlere sahip oldukları görülmüştür [3]. Çöp veya 'junk' DNA olarak adlandırılan genomun bu kısmı, insan genomundaki hareketli elementler olan transpozonları da kapsamaktadır [4]. Transpozonlar ilk kez 1940'lı yılların sonlarında mısır genomu üzerinde çalışmalar yapan Amerikalı bilim insanı Barbara McClintock tarafından keşfedilmiştir [5]. McClintock gerçekleştirdiği keşfin ardından 1983 yılında Nobel Fizyoloji veya Tıp ödülüne layık görülmüş ve transpozonların sanılanın aksine, işe

yaramayan DNA parçaları değil, önemli düzenleyici görevlere sahip yapılar olduğunu belirtmiştir [4].

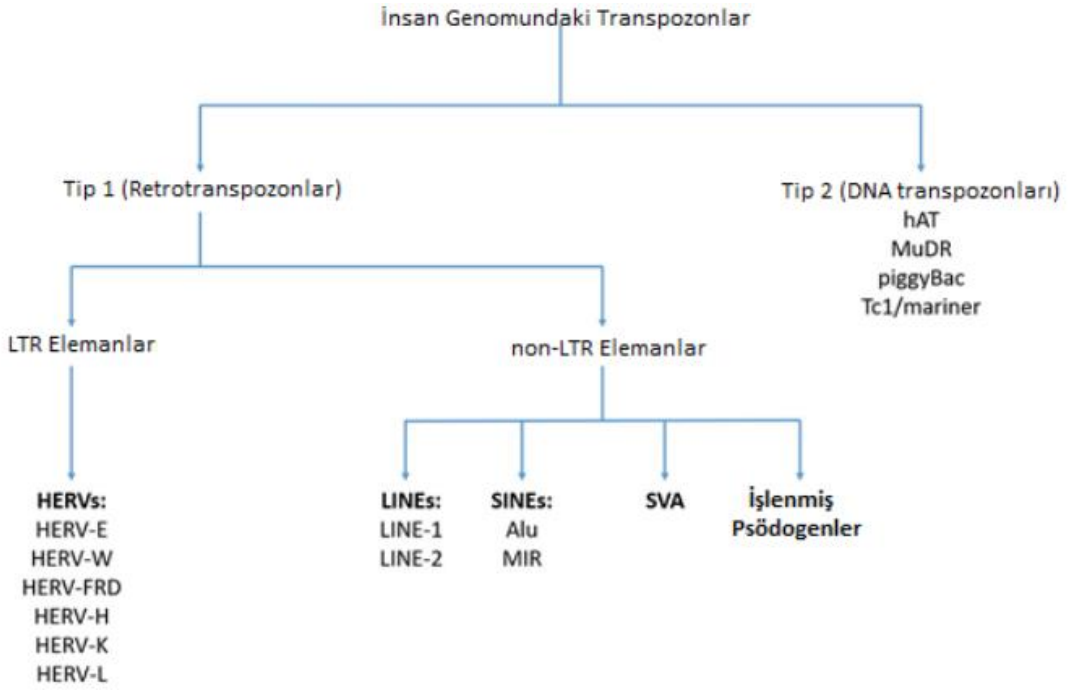
Transpozonlar, genomdaki konumlarını değiştirebilme yeteneğine sahip olan DNA parçalarıdır [6]. Transpozonların konum değiştirme hareketi, transpozisyon olarak adlandırılmakta ve transpozonlar, sahip oldukları transpozisyon yönteminin mekanizmasına göre iki alt grupta sınıflandırılmaktadır (Şekil 1). Bunlar, sınıf 1 olarak bilinen RNA transpozonları (retrotranspozonlar) ve sınıf 2 olarak bilinen DNA transpozonlarıdır (Şekil 2) [7]. Retrotranspozonlar, revers (ters) transkriptaz enzimi aracılığıyla, 'kopyala-yapıştır' mekanizmasıyla genomda yer değiştirirken; DNA transpozonları ise transpozaz enzimi aracılığıyla 'kes-yapıştır' şeklinde yer değiştirmektedir [8]. DNA transpozonlarının genomdaki hareketi, bir bölgeden ayrılıp başka bir bölgeye yerleşme şeklindedir. Bu nedenle genomdaki kopya sayısında herhangi bir artış meydana gelmez. RNA transpozonları olan retrotranspozonlar ise, genetik materyalleri olan mRNA'yı konak canlının transkripsiyon mekanizması aracılığıyla sentezleyerek, revers transkriptaz enzimleri ile bu mRNA'yı cDNA'ya (complementary DNA) dönüştürürler (Şekil 1). Bu transkripsiyon mekanizmasına retrotranspozisyon adı verilmektedir [9]. Retrotranspozisyon mekanizması ile genomda yer değiştiren hareketli elementler, genomdaki kopya sayısında artışa neden olmaktadır [10].



Şekil 1 RNA transpozonu (retrotranspozon) ve DNA transpozonunun hareket (transpozisyon) mekanizmaları [11]

Fig 1 Movement mechanisms of RNA transposon (retrotransposon) and DNA transposon [11]

Retrotranspozonlar ve DNA transpozonları kendi içlerinde alt sınıflara ayrılmaktadır [12]. Sınıf 1 olarak bilinen retrotranspozonlar, LTRs (Long Terminal Repeats- Uzun Terminal Tekrarlar) içerip içermemesine bağlı olarak 2 alt sınıfa ayrılmaktadır (Şekil 2). İnsan genomunun yaklaşık %45'i, LINE (Long Interspersed Nuclear Element- Uzun Serpiştirilmiş Nükleer Elementler) ve SINE (Short Interspersed Nuclear Element- Kısa Serpiştirilmiş Nükleer Elementler) gibi non-LTR retrotranspozonlardan ve DNA transpozonlarından meydana gelirken; %8'lik kısmı ise LTR retrotranspozonlar grubunda yer alan HERV dizilerinden (Human Endogenous Retrovirus - İnsan Endojen Retrovirüs) meydana gelmektedir [13]. Endojen retrovirüslerin her iki ucunda yer alan LTR'ler, endojen retrovirüsler ile LINE ve SINE gibi diğer retrotranspozonlar arasındaki temel farkı oluşturmaktadır [14].



Şekil 2 Transpozonların RNA ve DNA transpozonları olarak sınıflandırılması [12]

Fig 2 Classification of RNA and DNA transposons [12]

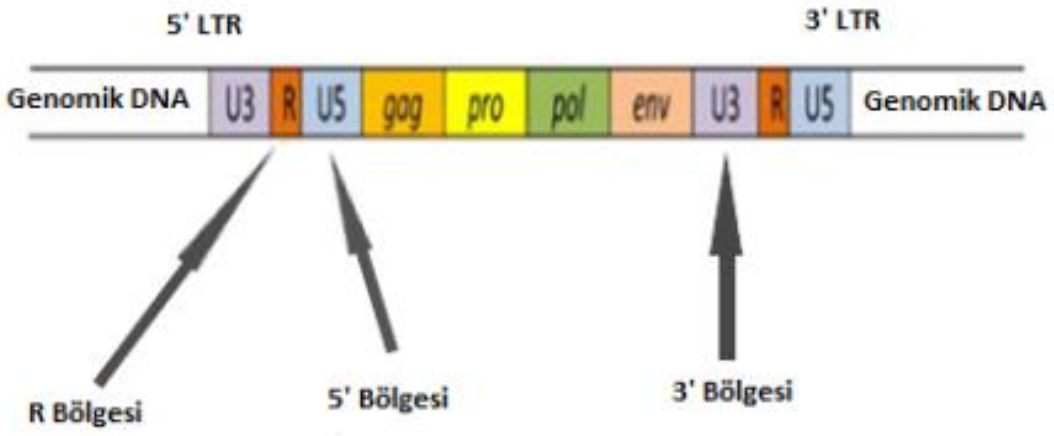
Transpozonlar, ilk defa 1940 yılında Babara McClintock tarafından keşfedildikten sonra birçok canlının genomunda yer aldığı ve genom obezitesine neden olmakla birlikte dizi varyasyonlarının konağın hızlı evrimine olanak sağladığı anlaşılmıştır. Bu derlemede, insan genomunda yer alan retrotranspozon ailelerinden biri olan insan endojen

retrovirüslerinin (HERV- Human Endogenous Retrovirus) sınıflandırılmasından, genom organizasyonuna olan etkilerinden ve hastalıklarla olan ilişkilerinden bahsedilecektir.

ERV (Endogenous Retroviruses- Endojen Retrovirüsü)

İnsan genomunun önemli bir kısmını oluşturan ERV, eksojen retrovirüslerden farklı olarak eşey hücrelerini etkilemekte ve bu nedenle nesiller boyunca aktarılmaktadır [15]. ERV'lerin, retrovirüslerin genoma entegrasyonu sonucunda oluştuğu bilinmektedir. ERV'lere ait olan yaklaşık 200.000 gen dizisi, insan genomuna entegre olmuş durumdadır. İnsan genomundaki endojen retrovirüs genlerinin bazıları, milyonlarca yıl süren evrimsel süreçte mutasyonlara uğrayarak anlatım yapma yeteneğini kaybetse de ERVK gibi bazı gruplar insan genomunda hâlâ anlatım yapmaktadır (Hurst & Magiorkinis, 2017). İnsan genomuna milyonlarca yıl önce entegre olan ERV'lerin; genomda, metilasyon ve histon deasetilasyonu gibi epigenetik mekanizmalar aracılığıyla susturulduğu bilinmektedir [17]. Diğer transpozonlar gibi ERV'ler de insan genomunun evrim sürecinde önemli rol oynamaktadır. ERV'lerin, insersiyon mutasyonları ve *cis* düzenleyici mekanizmalar ile transkripsiyonun aktivasyonu ve inhibisyonunu kontrol etmesi, insan genomu üzerindeki rollerine örnek olarak verilmektedir [18]. Ayrıca ERV genleri tarafından kodlanan bazı proteinlerin, gen anlatımının düzenlenmesini etkilediği gösterilmiştir [19].

ERV'lerin insan genomuna entegre olduğu bölgeler ve 5'- 3' uçlarında yer alan LTR dizileri incelenerek, insan genomunun evrim süreci hakkında bilgi edinilmektedir. LTR dizileri birer moleküler belirteç olarak değerlendirilmekte ve bu dizilerde meydana gelen mutasyonlar da göz önüne alınarak, ERV'lerin insan genomuna entegrasyon süreci üzerine çıkarımlar yapılabilmektedir [20]. İnsan genomundaki ERV kalıntılarının birçoğu, homolog rekombinasyonlar sırasında dizileri kaybolmuş olan izole LTR kopyalarından meydana gelmektedir [21]. ERV'lerin iki ucunda yer alan LTR dizilerinin 3' ucu (TG...) ile başlamakta ve 5' ucu (…CA) ile sonlanmaktadır (Şekil 3).



Şekil 3 Endojen retrovirüs dizi yapısının şematik gösterimi [24]

Fig 3 Schematic representation of the endogenous retrovirus sequence structure [24]

ERV'lerin yapısı büyük çoğunlukla 5'-LTR-*gag-pro-pol-env*-LTR-3' diziliminden meydana gelmektedir (Şekil 3). Bu dört gen (*gag*: gruba özgü antijen; *pro*: proteaz; *pol*: polimeraz; *env*: zarf) replikasyon için gerekli olan yapısal ve fonksiyonel proteinleri kodlamaktadır [13]. U3 bölgesi, transkripsiyonun başlangıcı için gerekli olan transkripsiyon faktörlerinin bağlandığı dizileri içerdiğinden, transkripsiyon, 5' ucundaki U3 bölgesinde başlamaktadır [25]. Transkripsiyon başlangıç noktaları 'GC/GT', bitiş noktaları ise 'AATAAA' dizilerini içermektedir. Endojen retrovirüslerin insan genomuna entegrasyonu sırasında bu bölge (U3) çeşitli mutasyonlara maruz kalarak, virüsün transkripsiyonel yeteneklerinin olumsuz etkilenmesine neden olmuştur [26]. 5' ucunda yer alan *gag* bölgesi, grup spesifik antijeni kodlamakta ve primer bağlanma bölgesini bulundurmaktadır. Primer bağlanma bölgesi, ters transkripsiyon işleminin başlamasında görev almaktadır [27]. *Gag* bölgesinden sonra yer alan *pol* bölgesi, endojen retrovirüslerin iç kısımlarında yer alan matriks, kapsid (protein kılıf-CA) ve nükleokapsid (NC) proteinlerini kodlamaktadır [28].

ERV'lerin insan genomuna entegrasyonu sürecinde, LTR dizilerinde ve *gag, pol, env* gibi gen bölgelerinde çeşitli mutasyonlar meydana geldiği saptanmıştır. Genellikle *gag* bölgesi daha fazla mutasyona maruz kalırken; *pol* ve *pre* gen bölgelerinin daha az mutasyona uğradığı görülmektedir [29]. Gen bölgelerinde meydana gelen mutasyonların çoğunun delesyon olduğu; delesyona uğramayan bölgelerde ise çerçeve kayması mutasyonları oluştuğu gözlenmiştir [27]. *Gag* ve *pol* genlerinin arasında yer alan *pro*

geni, proteinlerin yıkımından sorumlu olan proteinazı kodlamaktadır [22]. *Pol* gen bölgesi poliproteini kodlamakta ve ters transkriptaz ile integraz (IN) dizilerini barındırmaktadır. Zarf proteinlerini kodlayan *env* geni ise yüzey birimi (yüzey proteini-SU) ve transmembran proteinlerini (TM) kodlayarak, eksojen retrovirüslerin enfekte edeceği hücre tipinin belirlenmesinde rol almaktadır [23] .

İnsan Genom Organizasyonu (HUGO) insan genomundaki genleri protein kodlayan-kodlamayan genler ve psödogenler olarak adlandırmaya başladığında, endojen retrovirüsleri 'ERV' olarak isimlendirmeyi önermiştir [30]. Bu yeni isimlendirmeye göre endojen retrovirüsler, insanın ya da diğer farklı omurgalıların genomuna entegrasyonu göz önüne alınmadan 'ERV' olarak adlandırılmaktadır. ERV'lerin grup sembolleri güncellenirken 'Repbase' sembolleri kullanılmaktadır [31]. Görüldüğü üzere 'H' sembolü çıkarılarak grup ile alt grup sembolleri değiştirilmektedir (Tablo 1). Yeni isimlendirme yönteminin duyurulmasına rağmen eski isimlendirme yöntemi hala literatürde yaygın biçimde kullanılmaktadır [20].

Tablo 1 ERV'lerin yeni nomenklatüre göre grup sembolleri [31]

Table 1 Group symbols of ERVs by new nomenclature [31]

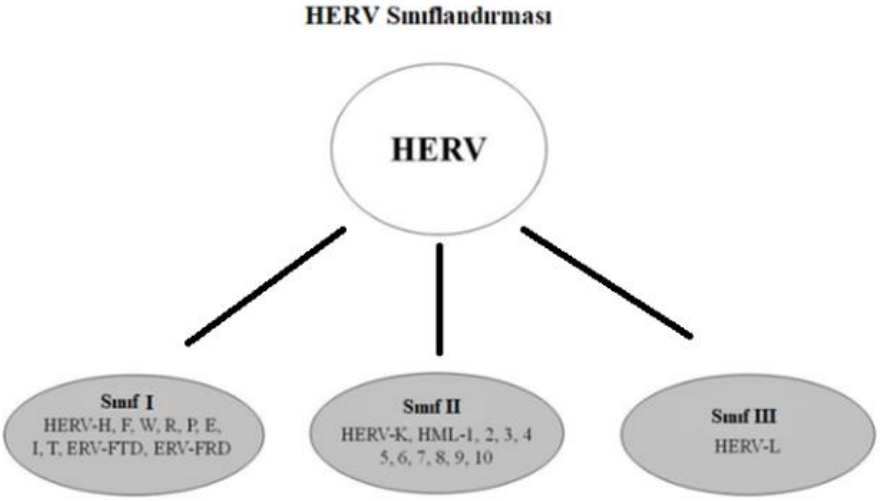
Repbase Grup Sembolü	**Yeni Nomenklatür Grup Sembolü**
HERVK	ERVK
HERVK3	ERVK3
MER50	ERVFRD
ERV3	ERV3
PABL-B	ERVPABLB
HERV-Rc1	ERVFC1
HERV17	ERVW
HERVS71	ERVS71
HERVH48	ERVH48
HERVH21	ERVFH21
HERVE	ERVE
HERV18	ERHV18
MER61	ERVMER61
HERVH	ERVH
HERV9	ERV9

Eksojen retrovirüsler, revers transkripsiyon mekanizması ile sahip oldukları RNA molekülünden cDNA sentezleyerek hedef konak organizmanın genomuna entegre olmaktadır. ERV'ler genoma entegre olduğunda ise, sahip oldukları *env* ve *pol* gibi gen bölgelerinin bazı kısımları mutasyonlar veya metilasyonlar gibi epigenetik

mekanizmalar nedeniyle işlevsiz hale gelmektedir [32]. ERV'ler ile eksojen retrovirüsler arasındaki bu farklılığın, insan genomuna entegrasyonları sırasında geçirdikleri uzun evrimsel süreçler nedeniyle olduğu düşünülmektedir [33]. ERV'ler ile eksojen retrovirüsler arasındaki bir diğer fark ise, ERV genomunun evrimsel süreçte daha az mutasyona maruz kalmış olmasıdır. Bu mutasyonlar nedeniyle çoğu gen bölgesi, anlatım yapma yeteneğini kaybetmektedir [16].

HERV (Human Endogenous Retrovirus- İnsan Endojen Retrovirüsü)

İnsan genomunun yaklaşık %8'lik kısmını oluşturan HERV'lerin insan hücrelerinde ilk kez tespit edilmesi 1970'li yıllara dayanmaktadır (Hurst & Magiorkinis, 2017). HERV'lerin milyonlarca yıl önce insan genomuna entegre olduğu ve hâlâ genomda varlıklarını sürdürdükleri tahmin edilmektedir [34]. Diğer hareketli genom elementleri (transpozonlar) gibi HERV'ler de insan genomunun evrim sürecinde önemli rol oynamaktadır. HERV'lerin genel olarak 3 sınıf altında toplanmaktadır (Şekil 4). Sınıf II'de yer alan insan endojen retrovirüs K (HERV-K), insan genomuna nispeten yakın bir zamanda entegre olan, transkripsiyonel aktiviteler bakımından genomdaki en aktif ERV'dir [35]. İnsan referans genomunda 120'nin üzerinde HERV-K insersiyonu saptanmıştır ve bu insersiyonların insanın yakın akrabası olan şempanze ve goril gibi kuyruksuz maymunlarda bulunmaması, HERV-K'nın insan genomuna entegrasyonunun yakın bir zamanda gerçekleştiğini destekler niteliktedir [36].



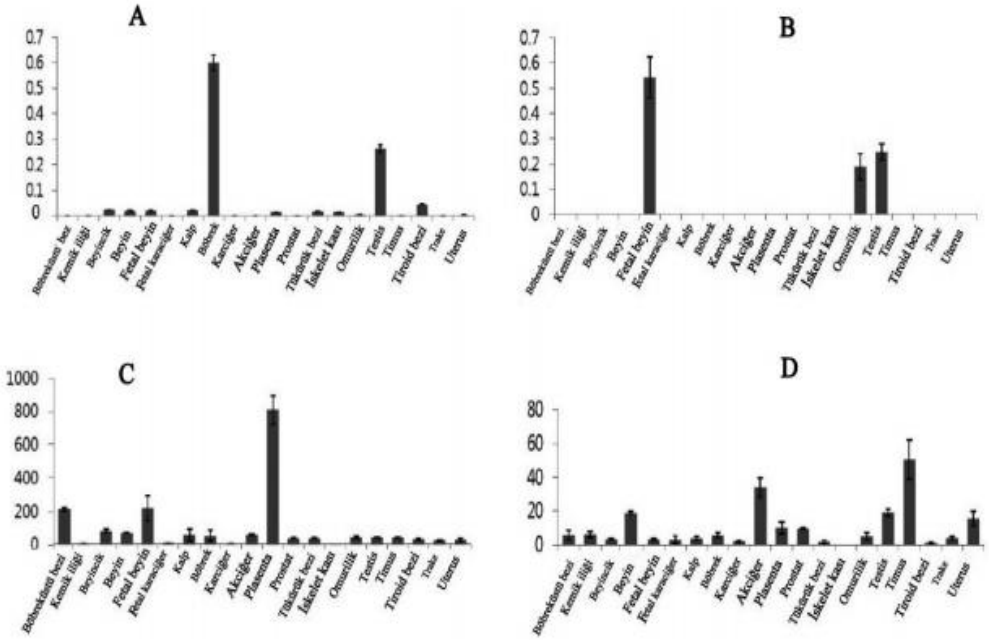
Şekil 4 HERV'lerin sınıflandırılması [37]

Fig 4 Classification of HERVs [37]

Sınıf 1 HERV'lerde meydana gelen mutasyonlar nedeniyle Sınıf 1'de yer alan HERV'lerin, Sınıf 2'de yer alan HERV'lerden daha düşük bir transkripsiyon aktivitesine sahip olduğu düşünülmektedir [38]. Sınıf 2 HERV'lere dahil olan HERV-K'nın diğer HERV'lere kıyasla daha az mutasyona maruz kaldığı bilinmekte ve bu nedenle HERV'ler arasındaki en yüksek transkripsiyonel aktiviteye sahip olduğu görülmektedir [36]. HERV-K grubunun gen anlatım analizleri incelendiğinde, deri, ovaryum ve meme kanserleri ile şizofreni gibi çeşitli hastalıkların ortaya çıkışında HERV-K'nın rol oynadığı görülmektedir [39]. Bu nedenle HERV-K'nın spesifik proteinleri ve mRNA'ları, bazı kanser vakalarının tanısında kullanılabilmektedir [40]. HERV-K, mutasyonları en iyi anlaşılmış HERV gruplarından biri olarak görülmektedir. HERV-K'nın insan genomunda yaklaşık 2500 LTR dizi kopyasına sahip olduğu bilinmekte ve bu dizilerde toplam 75 mutasyon saptanmaktadır [36].

İnsan Endojen Retrovirüslerin Hastalıklarla İlişkisi

Şimdiye kadar yapılan çeşitli çalışmalar sonucunda, endojen retrovirüslerin kanser, multipl skleroz (MS) ve şizofreni gibi çeşitli hastalıkların ortaya çıkmasında bazı rollerinin olduğu görülmüştür [31]. Farklı dokularda farklı anlatım oranlarına sahip olsalar da, endojen retrovirüslerin tüm insan doku ve hücrelerinde anlatım yaptığı bilinmektedir [43]. İnsan genomuna yakın bir zamanda entegre olan ve genomdaki en aktif endojen retrovirüs olan HERV-K'nın böbrek dokusunda, HERV-R'nin plasenta dokusunda, HERV-H'nin fetal beyin dokusunda, HERV-P'nin ise en yüksek seviyede akciğer dokusunda anlatım yaptığı, 20 farklı doku üzerinde yapılan gen anlatım analizleri çalışmasının sonucunda gösterilmiştir [27] (Şekil 5). İnsan vücudundaki farklı dokuların anlatım düzeylerinin bu şekilde birbirinden farklı seviyelerde olmasının, hücresel transkripsiyon faktörlerinin birbirinden farklı olan dokulara farklı şekillerde etki etmesinden kaynaklandığı düşünülmektedir [36].



Şekil 5 Farklı HERV'lerin dokulara göre anlatım analizleri. A: HERV-K, B: HERV-R, C: HERV-H, D: HERV-P [27]

Fig 5 Expression analyses of different HERVs according to tissues. A: HERV-K, B: HERV-R, C: HERV-H, D: HERV-P [27]

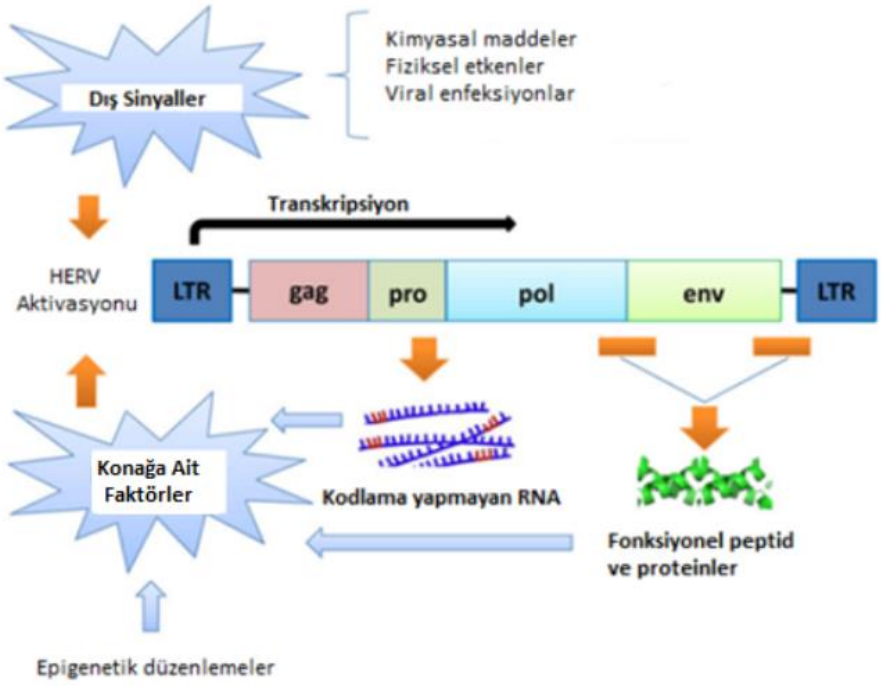
HERV'lerin Kanser ile İlişkisi

Kanser, hücrelerin kontrolsüz ve anormal bir şekilde çoğalması ile karakterize olan, genetik ve çevresel etkenler tarafından tetiklenebilen, toplumda sık görülen bir hastalıktır [44]. Sağlıklı hücreler, bölünme süreçlerini kontrol eden ve sınırlayan düzenleyicilere sahipken; kanser hücrelerinin bölünmesi kontrol altında tutulamamakta ve böylece anormal şekilde çoğalmış bir hücre topluluğu meydana gelmektedir [45]. HERV'lerin kanser ile olan ilişkisi uzun yıllardır araştırılmaktadır. Şimdiye kadar yapılan birçok çalışmada, kanser hücrelerinde HERV proteinlerine rastlansa da, HERV'lerin kanser gelişimine olan direkt etkisi hala tam olarak açıklanamamaktadır [46]. HERV'lerden kaynaklı kanserlerin, hipometilasyon aracılığıyla HERV dizilerinin aktive edilmesi, HERV'ler tarafından kodlanan *Np9* ve *Rec* gibi onkogenlerin anlatımı, mutasyonlar ile tümör baskılayıcı genlerin inaktivasyonu, homolog rekombinasyonlar, LTR'ler aracılığıyla büyüme faktörleri ve onkogenlerin transkripsiyonunun sağlanması gibi faktörler nedeniyle indüklendiği düşünülmektedir [47].

HERV'lerin 5' ve 3' uçlarında yer alan LTR dizileri promotör bölgeler içerdiğinden, HERV'lerin ve konak organizmanın transkripsiyonunu düzenleyebilmektedir [48]. LTR dizilerinin aktivasyonu bazı faktörler tarafından kısıtlanabilmektedir. LTR dizilerinin kombine edilerek tekli-LTR haline getirilmesi ve böylece replikasyon yeteneklerini kaybetmeleri LTR dizilerinin aktivasyonunu kısıtlayan faktörlere örnek olarak verilebilmektedir [16].

HERV-K'nın *env* proteinine spesifik olan monoklonal antikor 6H5 kullanılarak gerçekleştirilen immunohistokimya metodu ile deri kanseri hücrelerinde HERV-K *env* proteinleri tespit edilmiştir [49]. HERV-K-Mel antijeninin, kötü huylu (malign) deri kanseri hücrelerinin %85'inde anlatım yaptığı görülmektedir. Özellikle metastatik tümörlerde HERV-K *env* anlatımının, iyi huylu (benign) tümörlerdeki *env* anlatımından çok daha yüksek olduğu tespit edilmiştir [50]. HERV-K *env* proteinleri ayrıca teratokarsinom (testis tümörü), sarkom (yumuşak doku kanseri), mesane, meme ve over kanser hücrelerinde de gözlemlenmiştir [39]. HERV-K, HERV-H ve HERV-R gibi çeşitli HERV'lerin *env* genlerine ait mRNA'lar, primer meme kanseri hücrelerinde tespit edilmiştir [52; 53]. *Env* gen seviyelerinin adjuvan kemoterapi uygulaması sırasında azaldığı görülmektedir [50]. Ayrıca meme kanseri hastalarının %30'undan fazlasında anti-HERV-K ve anti-HERV-E antikorlarına rastlanmıştır. Nod-pozitif meme kanserine sahip hastalarda bu oranların, nod-negatif hastalara göre daha yüksek seviyelerde olduğu görülmektedir [54].

UV ışınları, sigara ve kimyasal maddeler gibi çevresel faktörlerin, kanser dokusundaki HERV proteinlerinin anlatımının artmasına neden olduğu düşünülmektedir (Şekil 6). Fiziksel ve kimyasal etkenlere ek olarak viral enfeksiyonların da HERV aktivasyonunu arttırdığı bilinmektedir. HIV-1 hastalarında HERV geni anlatımının sağlıklı bireylerden daha fazla olması bu duruma örnek olarak verilmektedir [55].



Şekil 6 HERV aktivasyonuna neden olan etkenler [26]

Fig 6 Factors causing HERV activation [26]

UV ışınları ve kimyasallar gibi dış etkenlerin yanı sıra, konağa ait faktörlerin de HERV aktivasyonunu etkilediği bilinmektedir (Şekil 6). DNA metilasyonu ve histon modifikasyonları gibi epigenetik mekanizmaların, transkripsiyon faktörlerinin ve sitokinlerin HERV aktivasyonunda rol aldığı çeşitli çalışmalarla gösterilmiştir [56]. Son dönemde yapılan çalışmalar, HERV demetilasyonunun kanserle olan ilişkisinin üzerinde durmaktadır. Bazı HERV'lerin DNA demetilasyonuna uğradığını ve bu durumun tümör gelişimine katkı yaptığı gösterilmiştir [58]. Baş-boyun kanseri hastalarından alınan dokuların DNA metilasyon analizleri yapıldığında, sağlıklı dokulardaki tüm HERV'lerin yoğun bir şekilde DNA metilasyonuna uğradığı görülürken, tümör dokusunda HERV-H'nin DNA demetilasyonuna uğradığı görülmektedir [59]. Benzer şekilde testis ve yumurtalık kanseri hastalarından alınan tümör dokuları incelendiğinde HERV'lerin belirgin şekilde DNA demetilasyonuna uğradığı görülmüştür [60], [61]. Elde edilen bu sonuçlar, anormal demetilasyonun anormal HERV anlatımına neden olabileceği görüşünü destekler niteliktedir [62].

Farklı bir epigenetik mekanizma olan histon modifikasyonlarının HERV'ler üzerindeki etkisinin, DNA metilasyonlarının etkisinden çok daha kompleks olduğu bilinmekle birlikte; histon modifikasyonlarının HERV anlatımlarının düzenlenmesindeki rolü tam

olarak aydınlatılamamıştır [63]. Buna rağmen, fare embriyonik kök hücreleri üzerinde yapılan bir çalışmada histon metilasyonlarının, embriyogenez sürecinde aktif olan endojen retrovirüsleri sessizleştirdiği görülmektedir [64]. Ancak histon deasetilaz inhibitörleri kullanılarak gerçekleştirilen çalışma sonucunda, HERV anlatımının kontrolü için histon asetilasyonunun gerekli olmadığı görülmüştür [65]. Böylece, farklı histon modifikasyonu mekanizmalarının HERV anlatımını farklı şekillerde etkilediği anlaşılmaktadır [66].

HERV aktivasyonunun, transkripsiyon faktörleri tarafından da düzenlenebildiği bilinmektedir [26]. HERV transkripsiyonu genellikle bir TATA kutusu içeren bir promotör tarafından başlatılmaktadır. HERV-K LTR'lerinin TATA kutusu görevi gördüğü ve transkripsiyonel aktivitesinin, Sp1 ve Sp3 transkripsiyon faktörleri tarafından düzenlendiği gösterilmiştir [57]. Sp transkripsiyon faktörleri ailesine dahil olan Sp1 ve Sp3 transkripsiyon faktörlerinin, hücre gelişimi, apoptozis ve karsinogenez gibi süreçlerde rol aldığı ve kanser hücrelerinde yüksek oranda sp1 ve sp3 anlatımı yapıldığı bilinmektedir [67]. Yapılan çalışmalar sonucunda, sp1 ve sp3 transkripsiyon faktörlerinin, kanser gelişiminde etkili olan *epidermal büyüme faktörü reseptörü (EGFR), vasküler endotelyal büyüme faktörü (VEGF)* ve *B-hücreli lenfoma 2 (BCL-2)* gibi onkogenlerin düzenlenmesinde rol aldığı görülmüştür [68].

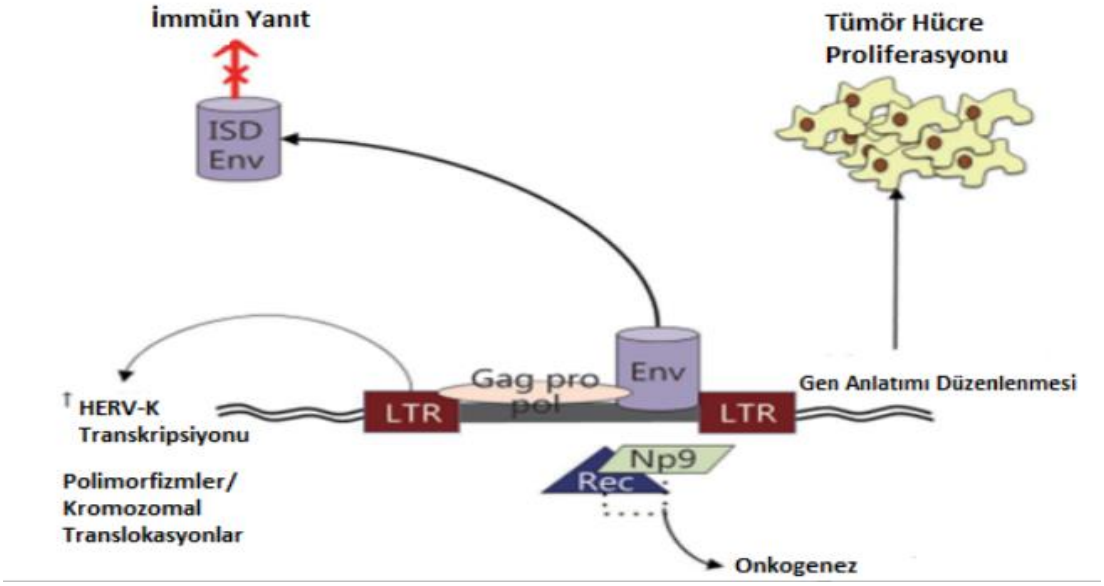
HERV'lerin birden çok mekanizma aracılığıyla kansere etki ettiği düşünülmektedir (Şekil 7). Bu mekanizmalardan ilkinde LTR dizileri rol oynamaktadır. LTR dizileri, retroviral gen transkripsiyonunu gerçekleştirmek için enfekte olmuş hücrelerin transkripsiyon faktörlerini kullanmaktadır [16]. Bu LTR dizilerinin aynı zamanda transkripsiyon faktörü görevi görerek, konak hücre genlerinin transkripsiyonunu arttırdığı ve böylece kontrolsüz hücre bölünmesine neden olduğu gösterilmiştir [26; 47].

HERV'lerin kansere etki mekanizmalarından ikincisi ise HERV-K gibi bazı HERV türlerinin *np9* ve *rec* gibi onkogenik etki gösterme potansiyeline sahip proteinler kodlamasıdır (Şekil 7). Bu proteinler transkripsiyon faktörleriyle etkileşime geçerek beta-katenin yolağı gibi bağışıklık yanıtın baskılanmasına neden olan yolakları aktive etmektedir [69]. Ayrıca tümör hücrelerinde genellikle aşırı derecede aktif olan MITF transkripsiyon faktörünün, HERV-K'nın LTR dizilerinin transkripsiyonal aktivitesi için gerekli olduğu görülmektedir [70].

HERV'ler tarafından indüklenen ve somatik hücrelerde gerçekleşen kromozomal translokasyon, HERV'lerin bir diğer kanser etki mekanizması olarak bilinmektedir (Şekil 7). Prostat kanseri hastalarında HERV-K ve E26 transkripsiyon faktörünün kombine olduğu ve HERV-K'nın insersiyon polimorfizmlerinin, sigara kullanmayan hastalarda akciğer adenokarsinomu görülme ihtimalini arttırdığı görülmektedir [71].

HERV'lerin son kanser mekanizması ise, bağışıklık yanıtının baskılanarak kanser oluşumu ve yayılmasına yol açmasıdır [48] (Şekil 7). Env proteinlerinin, bağışıklık yanıtının baskılanmasına neden olan bölgeler içerdiğini ve böylece tümör hücrelerinin immün sistemin takibinden kaçmasına yardım ederek kanser oluşumuna katkı sağladığı görülmektedir [72].

HERV'ler tarafından kullanılan bu karsinogenez mekanizmaları, bağışıklık sistemi virüslere karşı zayıf olan bireylerde çok daha riskli hale gelmektedir [73]. Kalıp tanıma reseptörleri (PRR), toll-benzeri reseptörler ve *RIG-1* gibi doğal immün yanıtta rol alan etmenlerde farklılaşmalar meydana geldiğinde oluşan potansiyel kanser riskinin, kontrolsüz HERV anlatımından kaynaklandığı düşünülmektedir [47], [74].



Şekil 7 HERV'lerin karsinogenez mekanizmaları [47]

Fig 7 Carcinogenesis mechanisms of HERVs [47]

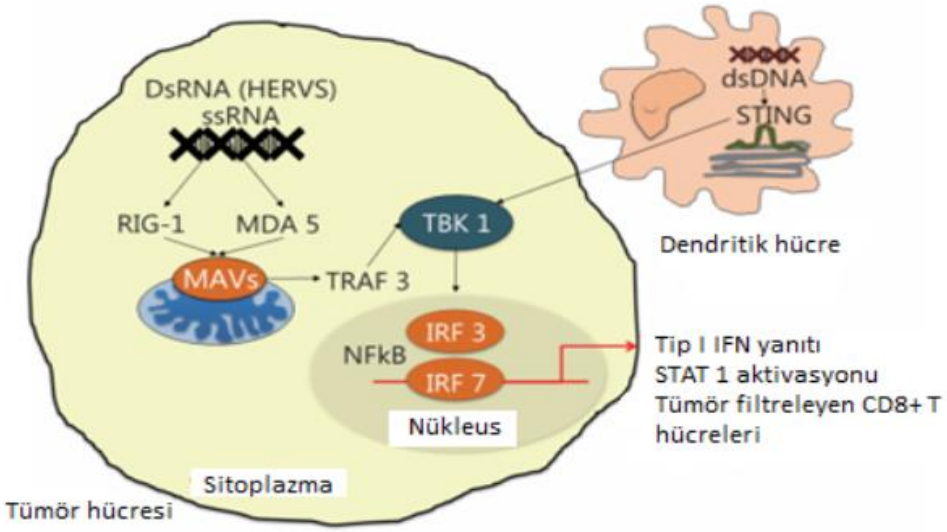
Kanser Tedavisinde HERV'lerin Kullanımı

Kanser hücrelerinde meydana gelen yüksek seviye HERV proteinleri anlatımı, immünoterapi için bir hedef niteliği taşımaktadır. HERV-K ve HERV-H gibi bazı

HERV türlerinin env genleri tarafından kodlanan proteinler immunojenik özelliğe sahiptir ve HERV'lere karşı humoral ve hücresel yanıtlar meydana getirdikleri tespit edilmiştir [47]. HERV-K'ya karşı oluşturulan antikorların, *in vitro* ve hayvan deneylerinde kanser gelişimini engellediği görülmektedir [52]. HERV'lerin retroviral bir kökene sahip olması, HERV antijenlerinin varlığının doğal bağışıklık yanıtını tetikleyerek organizmadaki anti-tümör aktivitenin artmasına neden olmaktadır [75]. Bu nedenle, kanser hücrelerinde yüksek oranda anlatımı gerçekleşen HERV proteinleri, kanser tedavisinde immün yanıt için bir hedef olarak görülmektedir [48].

Histon deasetilaz inhibitörleri ve CTLA-4 antikoru olan ipilimumab gibi kontrol noktası inhibitörlerinin kombinasyonundan meydana gelen yeni bir tedavi yaklaşımı, kanser tedavisinde kullanılmak üzere test edilmeye başlanmıştır [76]. Bu yaklaşım, histon deasetilaz inhibitörleri ve DNA metiltransferaz inhibitörleri aracılığıyla HERV gen transkripsiyonun tekrar aktive edilmesi temeline dayanmaktadır. HERV transkripsiyonunun tekrar aktive edilmesi ile immün yanıtın HERV antijenlerini hedef alması ve kanser gelişimini engelleyici yönde rol oynaması amaçlanmaktadır [47]. HERV anlatımı, sitozoldeki tip 1 interferon yanıtını, ikincil STAT1 aktivasyonu aracılığıyla aktive eden tek iplikli RNA zincirinin (*RIG1* VE MDA5) kalıp tanıma reseptörlerini aktive etmektedir [77]. Kalıp tanıma reseptörleri ligandlarına bağlanarak sinyal yolaklarını aktif hale getirmektedir. Sonuç olarak bu durum, interferon düzenleyici faktör 3 ve 7'yi (IRF-3 ve IRF-7) indükleyen ve interferon-beta (IFN-beta) üretimini sağlayan bir protein kinaz olan TANK-bağlayıcı kinaz 1 (TBK 1)'in aktivasyonuna neden olmaktadır. İnterferon-beta, STAT'ı aktive ederek CD8 T hücreleri tarafından gerçekleştirilen kanser hücresi tanınmasını arttırmaktadır [78] (Şekil 8).

Histon deasetilaz inhibitörleri ve kontrol noktası inhibitörlerinin kombinasyonundan meydana gelen bu tedavi yaklaşımı ile epigenetik ilaçlar ve immünoterapi arasındaki ilişki açıklanmaya çalışılmıştır. Histon deasetilaz inhibitörlerinin kullanıldığı hayvan modellerinde CD8 T hücrelerinin üretiminin ve anti-tümör aktivitenin arttığı gözlemlenmiştir [79]. Buna ek olarak, hipometile edici ajanların anti-CTLA 4 (ipilimumab) gibi kontrol noktası inhibitörleri ile kombinasyonunun da anti-tümör aktiviteyi arttırdığı görülmektedir [80], [81].



Şekil 8 HERV gen transkripsiyonun tekrar aktive edilme mekanizması [82]

Fig 8 Reactivation mechanism of HERV gene transcription [82]

Kansere karşı yakın zamanda gerçekleştirilen bu immünoterapi çalışmaları ile önemli sonuçlar elde edilmiştir. İpilimumab gibi kontrol noktası inhibitörlerinin cilt ve akciğer kanseri gibi kanser türlerinin tedavisinde etkili olduğu görülmektedir [71]. Bununla beraber, çeşitli ilaçların kombinasyonu ve radyoterapi ilavesi ile tedavinin verimliliğinin artabileceği tahmin edilmektedir [83]. Bu ilaç kombinasyonları, kanser hücrelerindeki HERV genleri tarafından kodlanan proteinleri hedef alan tümör karşıtı immün yanıtlar temel alınarak oluşturulmaktadır [84]. Kontrol noktası inhibitörlerini bloke eden ilaçlar ile epigenetik ilaçların kombinasyonu, henüz kesin bir tedavi yöntemi olarak adlandırılmasa da HERV kaynaklı kanser tedavisi için umut verici sonuçlar doğurmaktadır [47].

Son yıllarda yapılan çalışmalar, insan genomu ve HERV'ler arasındaki ilişkinin, memelilerin gelişiminde milyonlarca yıllık karmaşık ve çok yönlü bir ortak evrim oluşturduğunu göstermektedir. Bu uzun ilişkinin sonucunda, HERV etkileşimlerinin insan genomuna ve fizyolojisine çeşitlilik getirerek HERV'lerin zararlı etkilerinin dengelendiği düşünülmektedir. Özellikle HERV'lerin, insan antiviral bağışıklığının şekillendirilmesinde ve gelişiminde önemli katkılar sağladığı görülmektedir. Bununla birlikte, çeşitli kanser tiplerinde HERV anlatımının gözlemlenmesi, sağlıklı hücrelerde HERV anlatımının olmaması nedeniyle HERV ürünlerine karşı bağışıklık sisteminin manipüle edilerek kanser hücrelerini hedef alması sağlanabilmektedir. Bu da kanser

tedavisinde yeni tedavi stratejilerinin geliştirilmesine olanak sağlamaktadır. Son dönemde kanser tedavisinde immünoterapinin diğer ilaçlar ya da radyoterapi ile kombine edilmesi ile etkinliğinin arttırılabileceği gösterilmiştir. Deneysel olarak ortaya konan kanser hücrelerindeki HERV ürünlerinin hedeflendiği immünoterapik tedavi kombinasyonları kanser tedavisinde umut verici yaklaşımlar olarak kabul görmektedir.

Kısaltmalar

ERV: Endojen Retrovirüs; HERV: İnsan Endojen Retrovirüs; XRV: Eksojen Retrovirüs; LTR: Uzun Terminal Rekrarlar; LINE: Long Interspersed Nuclear Element; SINE: Short Interspersed Nuclear Element; MS: Multipl Skleroz; GAG: Gruba Özgü Antijen; Pro: Proteaz; *Pol*: Polimeraz; *Env*: Zarf; CA: Kapsid; NC: Nükleokapsid; PBS: Primer bağlanma bölgesi; HIV: Human Immunodeficiency Virus; cDNA: Tamamlayıcı DNA; mRNA: Haberci DNA; SU: Yüzey birimi; TM: Transmembran proteini; IN: İntegraz; UV: Ultra viyole; STAT1: Signal Transducer and Activator Of Transcription 1; *RIG1*: Retinoic Acid-Inducible Gene; MDA5: Melanoma Differentiation-Associated Protein 5; IRF-3: Interferon Düzenleyici Faktör 3; IRF-7: Interferon Düzenleyici Faktör 7; IFN-beta: İnterferon-beta; CD8 T: Sitotoksik T Hücreleri; TBK 1: TANK Bağlayıcı Kinaz; CTLA 4: Cytotoxic T-Lymphocyte-Associated Protein; HUGO: İnsan Genom Organizasyonu; *EGFR*: Epidermal Büyüme Faktörü Reseptörü; *VEGF*: Vasküler Endotelyal Büyüme Faktörü; *BCL-2*: B-hücreli Lenfoma 2; SP: Specificity protein

Destekleyen Kuruluş

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Genetic Analysis Related To Organized Genetic Changes in Potato And Processed Potatoes

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