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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 [1-2]. This substance production has began centuries ago; therefor several hypothesises illustrates how the first synthesis of yoghurt took place through the course of history [3]. 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. [4] 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

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specific standard. [4] Yoghurt 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 [5-6]. 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 [7].

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 [8-9]. *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 [10]. *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 [11]. 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 [12]. Milk components; it is broken down into carbonyl compounds, nonvolatile acids, and volatile acids [13]. Acetaldehyde, acetone, acetoin, diacetyl, and acetate are a few examples. These contribute to the distinctive flavor of yoghurt [14-15].

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 [16]. 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, [17] 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 [18]. Studies have observed that the polyphenols in pinecones are beneficial for human health [18-19-20]. According to Yi et al.'s (2017) research, pinecone possesses anticancer and antioxidant effects due to the different polyphenols found in it [20-21]. *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* were found in both yoghurt and pinecone in research conducted by Bostan et al. (2017) [22]. 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 [23]. 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 [22].

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 [24].

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) [25]. 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 [26].

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 [26].

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 [27].

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.

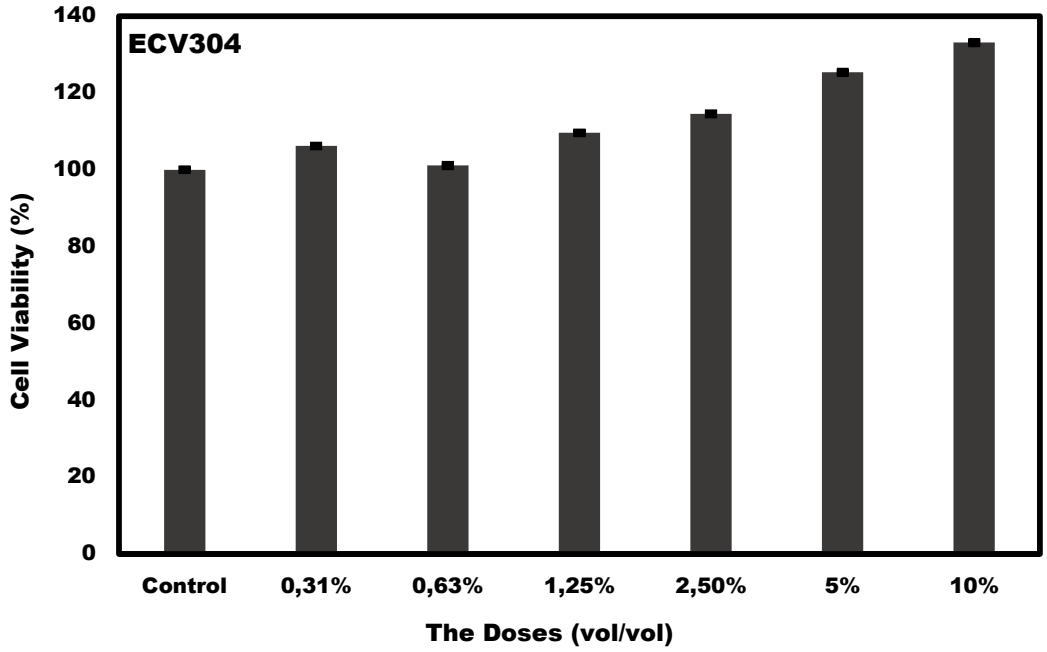

Fig 1 Cell viability results using MTT assay for ECV304 cell line (n= 6)

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.

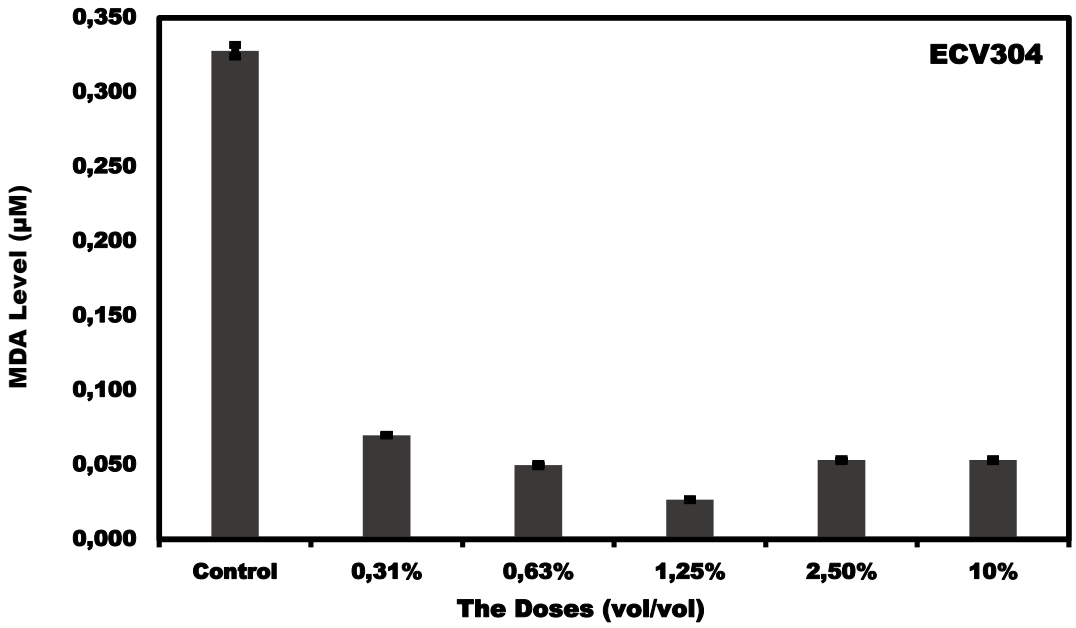

Fig 2 MDA levels affected by various concentrations of pinecone yoghurt in ECV304 cell line (n= 5)

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.

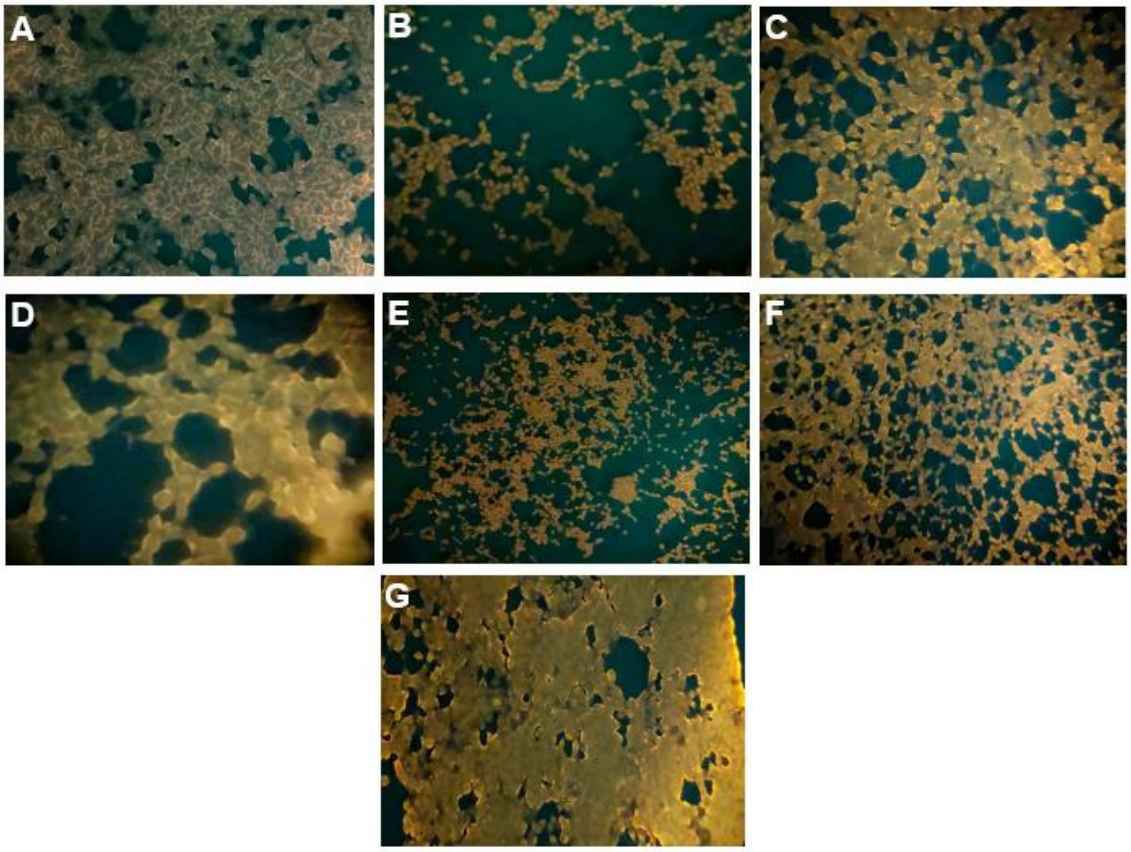

Fig 3 Fluorescent microscopic image of ECV304 cell line; control (A), treated with pinecone yoghurt at a concentration of 0,31% (vol/vol) (B), treated with pinecone yoghurt at a concentration of 0,63% (vol/vol) (C), treated with pinecone yoghurt at a concentration of 1,25% (vol/vol) (D), treated with 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)] [25]. 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 [28-29]. 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 [30]. 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 [22-31]. 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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