Determining the cytotoxic effect potential of ozonated hazelnut oil

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Abstract

Although the use of hazelnut oil is becoming ever more common, the ozonated form of this oil has never been obtained before. Ozonated vegetable oils are generally preferred in ozone therapy as they are easy to apply and obtain. In this study, the density, viscosity, peroxide and iodine values of hazelnut oil obtained by being treated with ozone in different periods at a flow rate of 7-8 were determined and compared with its commercial form, *i.e.* refined hazelnut oil. In order to determine whether ozonated hazelnut oil can be safely used, its cytotoxic effect potential was assessed by performing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium assay on three different cell lines. For this purpose, the viability at five different concentrations of each sample was calculated after 48 and 72 hours of incubation in the H1299 lung cancer, human umbilical cord vein endothelial cells and A549 cell line. As a result, it was found that hazelnut oil applied to three different cell lines and in five different concentrations (100/50/25/12.5/6.25µg/mL) did not have any cytotoxic effects. Furthermore, it was also revealed that the samples did not have a toxic effect due to treatment with ozone in comparison to refined ozonated hazelnut oil. Therefore, this study has shown that ozonated hazelnut oil can be used as an alternative to refined hazelnut oil without exerting any toxic effects due to treatment with ozone. In light of this information, it was concluded that hazelnut oil can be safely used after treatment with ozone without having any cytotoxic effects and that it can be produced as a functional oil providing both the pharmacological and cosmetic effects of ozone.

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Introduction

Plants have been used since the beginning of human existence in order to improve standards of living and for human health.[1] Among other countries that are rich in flora, Turkey is of great importance in terms of its plant diversity. Besides being among the top countries in terms of plant diversity and use, Turkey is also rich in fruit farming. One of many fruits that are grown in Turkey is the hazelnut, which is considered very valuable traditionally and of great importance from an economic aspect. Turkey has the most suitable conditions for hazelnut production and farming, wherein 74.8% of the hazelnut production areas in the world are located in Turkey, and 72.5% of this area is in the Black Sea Region. The hazelnut (*Corylus L.*) has 12 or 20 species, however *Corylusavellana* (common hazel), *Coryluscolurna* (Turkish hazel) and *Corylusmaxima* (filbert) are the species that are cultivated in Turkey.[2] The homeland of this cultivated plant is Turkey.The consumption of hazelnut oil, which has a chemical composition very similar to that of olive oil, has been significantly increasing in recent years. Hazelnut oil contains a high amount (82-84%) of oleic acid.[3] This is followed by linoleic (9-11%), palmitic (4%), and stearic acid (1.5-2.3%). Hazelnut oil is used in meals, fried dishes and salads as a replacement for other vegetable oils.[4] The reaction between ozone and vegetable oils takes place with carbon-carbon double bonds in unsaturated fatty acids which can be found in vegetable oils.[5] Ozonated vegetable oils have a much more complex structure compared to natural vegetable oils.[6] Compounds such as hydroperoxides, ozonides, aldehydes, peroxides, diperoxides and polyperoxides are obtained as a result of the interaction between ozone and vegetable oils.[7,8] These oxygenated products are sensitive to many biological activities. The effects of ozonated vegetable oils against bacteria and fungi are also closely related to their use in food, cosmetics and pharmaceutical industries and such fields.[9] In particular, ozonides and peroxides provide great benefit in medicine due to their antiseptic effects.[9] For this reason, in this study the toxic effects of the obtained ozonide intermediate products were determined and it was shown that these products could be safely used as a functional food.

This study investigated whether ozonated hazelnut oil can be a more functional product in comparison to the existing refined hazel-

nut oil by combining the medical and pharmacological properties of ozone with vegetable oils, as well as the toxicity potential due to active intermediate products obtained during treatment with ozone.

Materials and Methods

Obtaining the ozonated hazelnut oil

The oils used in this study were treated with ozone with the aid of an ozone generator at Düzce University Chemistry Laboratory, which was isolated from light, by providing ozone gas at a flow rate of 7-8 through 200 mL of oil at room temperature for 120 minutes. The oil was then mixed at 1000 rpm with a magnetic stirrer during treatment with ozone. The density, viscosity, peroxide and iodine values of the ozonated oils obtained in different periods, *i.e.* 60 and 120 minutes, were determined and the results are provided in Table 1. The produced oils were kept at 4°C throughout the procedure.

Growing the cell strains and cell counts

The H1299 lung cancer cell line, A549 cell line and human umbilical cord vein endothelial cells (HUVEC) were used in this study and prepared and kept at -80°C. They were then placed into the medium and centrifuged. The pellet was solubilized in the medium after the centrifuge process. A 5mL medium was placed in 25cm^2 culture plates. The plates were then left at 37°C in a 5% CO_2 environment for incubation.Right after the trypsinization took place, the mediums of the cells were removed by aspiration. After aspirating 1% of DPBS, 0.5mL of trypsin-EDTA solution was added in order to separate the cells from the culture medium and the cells were left for incubation in a 5% CO_2 environment at 37°C for four minutes. Afterwards, microscopic examinations were performed. 5mL of medium was added to this and the mixture was placed into a Falcon. The supernatant was removed after performing centrifuge. 5mL of medium was added and 7mL of cell suspensions were transferred into the medium and cultured at 37°C in a 5% CO_2 environment.[10,11] In order to count the cells, 10µL of the cell suspension obtained after trypsinization was added to a 0.5mL tube and an equal amount of 0.5% trypan blue was added to this and mixed. 15µL of the final solution was placed on a Thoma slide in an inverted microscope and the cells were counted. The obtained figures were multiplied by the dilution coefficient and the amount of cells in 1mL of medium was calculated.[11]

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide viability method

The cytotoxic effects were determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay, which is one of the test methods for evaluating cytotoxicity based on enzymatic activity. The MTT viability test is a colorimetric and affordable method that is used in measuring cell viability, proliferation or cytotoxicity. In this method based on colorimetric measurements, the main parameter is the metabolic activity of viable cells. The method is based on the basic principle of cleavage of the tetrazolium ring of MTT.

Statistical analysis

The results of the study were analyzed using the SPSS 21 (IBM) statistics software package. The data was evaluated using the Two-Way ANOVA Test. The values were analyzed using the Post-Hoc Tukey Test with a 95% confidence interval and the differences between the groups were determined.

Results

Initially for the MTT assay, when A549, H1299 and HUVEC cells covered at least 80% of the cell culture flask, the mediums were removed from the environment. Trypsin-EDTA solution was used in order to move the cells away from the flask base that they adhered to and away from each other. A549, H1299 and HUVEC cells were then counted and placed in the wells of a 96-well cell culture plate in a manner to include approximately 5000 cells per well, and left for incubation at 37°C, in an incubator with 5% CO_2 for 24 hours. Five different ozonated hazelnut oil samples at five different concentrations were each applied to the cells in an amount of 100µL with three repetitions and the cells were left to incubate for 48 hours. As a control, Triton X-100 run for three repetitions was used for maximum inhibition, no material was applied, and only the wells that contained cells MO and 5-Fluorouracil were placed. After incubation, 10µL of MTT solution was added to all wells and incubated for four hours at 37°C in an incubator that contained 5% CO_2. A microscope was used in order to see whether formazan crystals were formed in cells that were exposed to the MTT solution, and upon detecting the formation, sodium dodecyl sulfate solution was used to solubilize the crystals and the intensity of the formed color was measured with a spectrophotometer set at 570 nm wavelength. Absorbance values obtained from the measurement were used in calculating the viability percentage and the following formula was used.

Viability % = [100 × mean absorbance of the cells that were treated with ozonated hazelnut oil / absorbance of the control cells that were not treated with the sample (MO)]

The values obtained after applying the MTT method were evaluated. The viability rates are shown in the following graphs.

When comparing the viability rates in three different cell lines and at five different concentrations after incubating the non-

Table 1. Physical and chemical properties of the ozonated hazelnut oils.

Code of Sample	Samples	Density (g/mL, 25°C)	Viscosities (cpt, 25°C)	Peroxide (mEqO_2/kg)	Iodine Value
(1)	Refined Nut Oil	0,914	65,0	3,67	85,13
(2)	7-8 debi, 60 minutes ozonated non-refined nut oil	0,912	66,3	28,07	87,18
(3)	7-8 debi, 120 minutes ozonated non-refined nut oil	0,922	95,6	246,47	66,43
(4)	7-8 debi, 60 minutes ozonated non-refined nut oil with hexane extraction	0,918	77,8	160,16	80,08
(5)	7-8 debi, 120 minutesozonated non-refined nut oil with hexane extraction	0,925	92,0	301,45	53,17

ozonated refined hazelnut oil (Sample 1) for 48 hours, as seen in Figure 1A, the highest viability rate was observed at a 6.25µg/mL concentration and this concentration provided the highest viability in the HUVEC cell line. As seen in Figure 1B, it was also found that the highest viability rate after 72 hours of incubation was seen in the H1299 cell line at a 100µg/mL concentration.

According to the result of the statistical analysis based on cell lines, concentrations and incubation periods between Sample 1 (control) and other groups (samples 2, 3, 4, 5) among the studied sample groups (1 (control), 2, 3, 4, 5), there was no significant difference between the groups (P>0.05). As a result of the comparison between unrefined hazelnut oil ozonated for 60 minutes at a flow rate of 7-8 and obtained by hexane extraction, the control and other unrefined ozonated samples, it was found that there was a statistically significant difference between viability percentages according to the concentrations after 48 hours of incubation in the HUVEC cell line (P<0.05).

According to the result of the statistical analysis comparing the viability rates between the groups, Sample 5 had significantly different viability rates compared to the other groups and the viability percentage in Sample 5 was significantly higher than the other groups (P<0.05).

According to the Two-Way ANOVA Statistical Analysis comparing the groups with 72 hours of incubation, it was statistically shown that there was no significant difference between the control group (1) and other groups (2, 3, 4, 5) (P>0.05).

When comparing the viability rates in cell lines after 48 hours of incubation of Sample 2 unrefined hazelnut oil that was ozonated for 60 minutes at a flow rate of 7-8, as seen in Figure 2A, the highest viability rate was observed at a 12.5 µg/mL concentration in the HUVEC cell line. As seen in Figure 2B, it was also found that the highest viability rate after 72 hours of incubation was seen in the HUVEC cell line at a 12.5 µg/mL concentration (Table 2).

When analyzing the viability rates in cell lines after 48 hours of incubation of the unrefined hazelnut oil (Sample 3) that was ozonated for 120 minutes at a flow rate of 7-8, as seen in Figure 3A, the highest viability rate was observed at a 100 µg/mL concentration in the H1299 cell line. As seen in Figure 3B, it was also found that the highest viability rate after 72 hours of incubation was seen in the A549 cell line at a 12.5 µg/mL concentration.

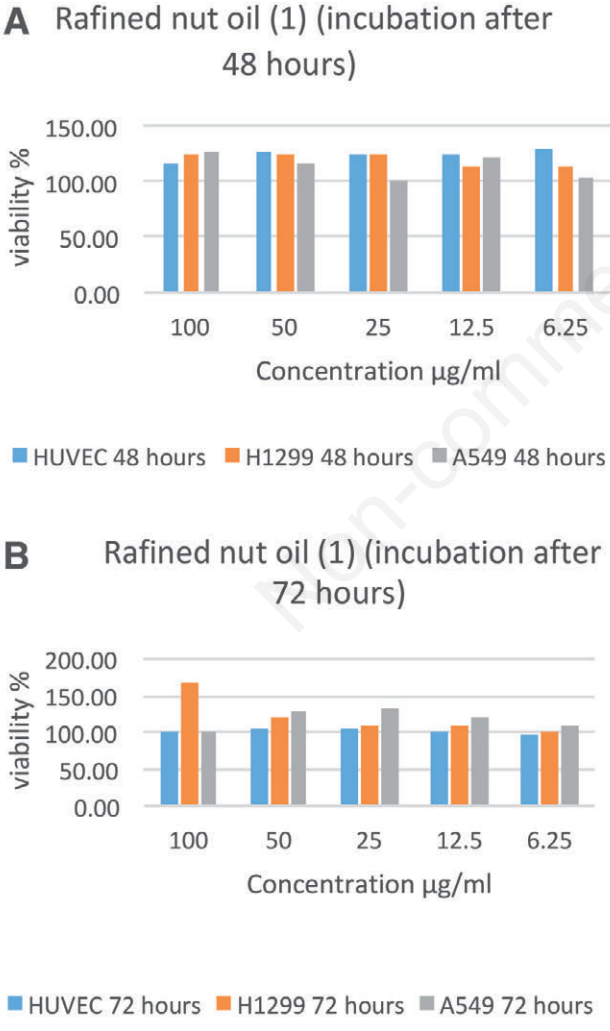

Figure 1. Viability rates of the cell lines after 48-72 hours of incubation of the non-ozonated refined hazelnut oil (Sample 1). A) Rafined nut oil (1) incubation after 48 hours; B) Rafined nut oil (1) incubation after 72 hours.

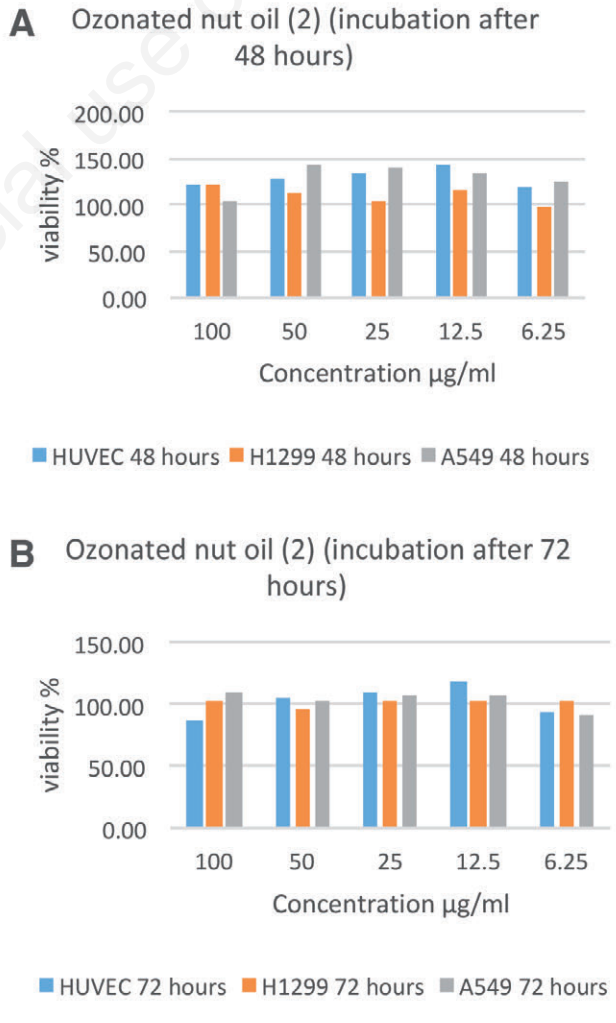

Figure 2. Viability rates of the cell lines after 48-72 hours of incubation of the unrefined hazelnut oil (Sample 1) ozonated at a 7-8 flow rate for 60 minutes. A) Ozonated nut oil (2) incubation after 48 hours; B) Ozonated nut oil (2) incubation after 72 hours.

When analyzing the viability rates in cell lines after 48 hours of incubation of Sample 4 (unrefined hazelnut oil that was ozonated for 60 minutes at a flow rate of 7-8 and obtained by hexane extraction), as seen in Figure 4A, the highest viability rate was observed at a 12.5 µg/mL concentration in the HUVEC cell line.

Analyzing the viability rates in cell lines after 72 hours of incubation of Sample 4 (unrefined hazelnut oil that was ozonated for 60 minutes at a flow rate of 7-8 and obtained by hexane extraction), as seen in Figure 4B, the highest viability rate was observed at a 6.25 µg/mL concentration in the HUVEC cell line.

When analyzing the viability rates in cell lines after 48 hours of incubation of Sample 5 (unrefined hazelnut oil that was ozonated for 120 minutes at a flow rate of 7-8 and obtained by hexane extraction), as seen in Figure 5A, the highest viability rate was observed at a 100 µg/mL concentration in the H1299 cell line.

When analyzing the viability rates in cell lines after 72 hours of incubation of Sample 5 (unrefined hazelnut oil that was ozonated for 120 minutes at a flow rate of 7-8 and obtained by hexane extraction), as seen in Figure 5B, the highest viability rate was observed at a 6.25 µg/mL concentration in the H1299 cell line.

According to the viability data obtained from MTT assays, it was found that Sample 5 dropped the viability rate in the H1299 cell line down to 79% after 48 hours of incubation. In Sample 3, however, it was seen that the highest viability rate was in the H1299 cell line, even at the highest concentration. Samples 2-4 showed similar viability rates as the non-ozonated refined hazelnut oil sample, *i.e.* control group, in the HUVEC cell line. According to these results, it was found that samples 2-4 of the obtained ozonated hazelnut oils showed almost the same viability rates at similar concentrations compared to the control sample, and samples 3-5 of the ozonated hazelnut oil lowered viability rates, even at the highest concentrations, by exhibiting a cytotoxic effect on cancer cell lines.

Discussion and Conclusions

Ozonated hazelnut oil samples that were ozonated at a flow rate of 7-8 for different periods and commercially used non-ozonated refined hazelnut oil were used within the scope of this study. After treatment with ozone, fatty acids with double bonds within the oil were cleaved and this cleavage increased with fluctuations in parallel to the duration of treatment with ozone. The ozonization of double bonds increased the density significantly, while viscosity also exhibited a parallel increase. Peroxide formation was increased due to active oxygen and peroxide values reached very high figures. The change in iodine value is the strongest indicator that ozonization took place. The iodine value dropped in parallel to the duration of ozonization. The decrease in iodine value indicates that the number of double bonds is reduced. Within the scope of this study, the toxic effect potential of the ozonization duration, the ozonization itself and the commercial non-ozonated refined hazelnut oil as the control group were evaluated and it was investigated whether new ozonated hazelnut oil could be a functional alternative for commercially used refined hazelnut oil (Çotanak).

When reviewing the previously conducted studies, no studies were found in the literature. which examined the cytotoxic activity potential of ozonated hazelnut oil. Ozonated vegetable oils are commonly used in the food, cosmetics and pharmaceutical industries due to their high potential for therapeutic, antibacterial and

Table 2. All cell lines after MTT, incubation periods, concentrations and viability rates.

Sample codes		Human umbilical cord vein endothelial cells		H1299		A549	
		48hours	72hours	48hours	72hours	48hours	72hours
(1)	100 µg/mL	116,99	99,67	123,68	168,77	126,88	99,40
	50 µg/mL	127,45	104,14	125,21	119,53	116,60	128,74
	25 µg/mL	123.09	104,34	123,82	109,36	102,11	132,37
	12,5 µg/mL	125,38	101,93	113,51	109,24	121,34	121,99
	6,25 µg/mL	130,28	95,80	114,35	101,75	104,48	109,30
(2)	100 µg/mL	122,11	86,59	121,87	102,69	102,90	110,02
	50 µg/mL	128,87	104,80	112,26	96,96	142,42	102,17
	25 µg/mL	134,86	109,87	103,76	102,81	139,39	107,85
	12,5 µg/mL	143,57	119,35	114,90	102,46	134,52	107,97
	6,25 µg/mL	118,95	93,20	97,77	103,74	124,90	92,87
(3)	100 µg/mL	127,45	101,20	143,18	112,51	136,10	92,87
	50 µg/mL	122,66	101,20	105,71	88,07	135,57	105,07
	25 µg/mL	127,45	105,54	101,53	99,42	123,98	102,90
	12,5 µg/mL	122,88	100,20	110,31	92,16	110,80	114,86
	6,25 µg/mL	127,89	91,99	96,94	96,14	113,18	105,92
(4)	100 µg/mL	160,24	110,81	111,28	93,45	114,49	104,47
	50 µg/mL	220,08	230,96	107,39	122,34	127,14	91,18
	25 µg/mL	203,91	256,90	107,25	114,27	136,36	118,72
	12,5 µg/mL	242,82	254,39	130,82	120,70	128,59	120,65
	6,25 µg/mL	204,60	268,48	127,75	114,15	133,60	108,82
(5)	100 µg/mL	116,34	104,00	165,74	139,65	129,64	116,55
	50 µg/mL	112,09	107,20	107,80	114,39	132,15	116,18
	25 µg/mL	126,15	106,94	101,81	111,35	119,37	106,40
	12,5 µg/mL	125,28	103,07	112,67	110,76	109,09	112,92
	6,25 µg/mL	140,42	104,67	79,11	91,23	111,99	96,26

fungicide effects.[5,12-14]As a result of its reaction with oils, ozone gas is stabilized between carbon-carbon bonds contained within unsaturated fatty acids according to the Criegee mechanism.[15,16] Therefore, ozonated oils can remain stable for 2 years at 4°C.[17] In studies concerning ozonated olive oil, it was found that ozonated olive oil has various biological benefits guided by basic antioxidants.[18] Moreover, a cytotoxicity study was conducted by applying ozonated olive oil on L929 mouse fibroblast cells in vitro. Within the scope of the study, it was found that applying ozone alone affected cell viscosity and therefore resulted in significantly high cell death, wherein it was indicated that the duration of ozone treatment affected this viscosity. Ozonated oil was mixed with glycerol in order to reduce viscosity. According to the results of the study using the MTT method within this context, it was found that ozonated olive oil had a positive effect on cell viability and it was found to be statistically significant.[19]

According to another study on mice, the effect of ozonated sesame oil was investigated on collagen fibers and it was found that the fiber regeneration period was shortened as a result of applying ozonated sesame oil to damaged collagen fibers.[20] In our study, ozonated hazelnut oils were analyzed in HUVEC, H1299 and A549 cell cultures using the MTT method and it was found that the oil did not have any toxic effects. According to the results of our study which evaluated the viability rates, ozonated oils kept the viability rate at higher levels in comparison to non-ozonated refined oils.

Cytotoxicity tests provide information on the interaction of a substance or sample whose toxic effect is not known and provide a basis for the subsequent animal experiments or clinical trials. The main principle of a cytotoxicity study is to determine the amount of living/dead cells and these tests are commonly used in in vitro toxicology studies. In our study, the cell viability rates of these newly synthesized ozonated hazelnut oil samples have been identified using the MTT assay, which is a colorimetric method. It was found that none of the samples numbered as Sample 1 (non-ozonated refined hazelnut oil), Sample 2 (unrefined hazelnut oil ozonated for 60 minutes at a flow rate of 7-8), Sample 3 (unrefined hazelnut oil ozonated for 120 minutes at a flow rate of 7-8), Sample 4 (unrefined hazelnut oil ozonated for 60 minutes at a flow rate of 7-8 and obtained by hexane extraction), and Sample 5 (unrefined hazelnut oil ozonated for 120 minutes at a flow rate of

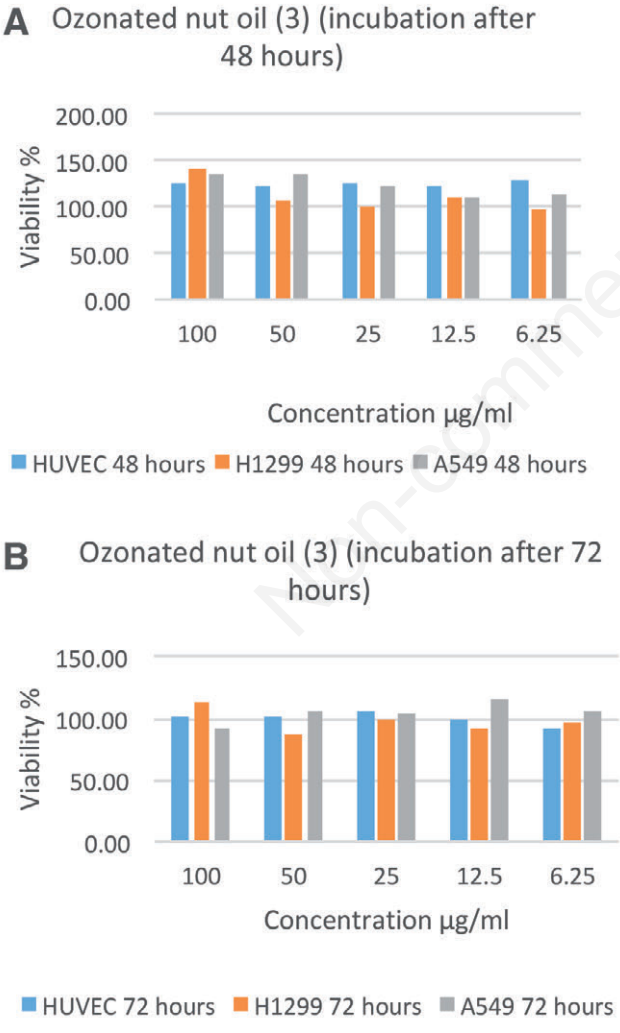

Figure 3. Viability rates of the cell lines after 48-72 hours of incubation of the unrefined hazelnut oil (Sample 3) ozonated at a 7-8 flow rate for 120 minutes. A) Ozonated nut oil (3) incubation after 48 hours; B) Ozonated nut oil (3) incubation after 72 hours.

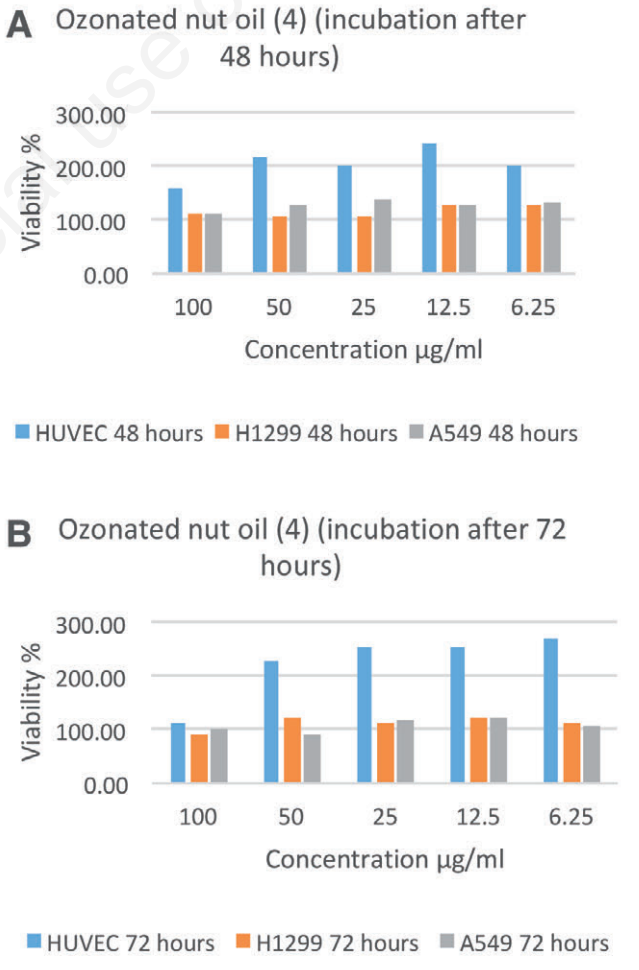

Figure 4. Viability rates of the cell lines after 48-72 hours of incubation of the unrefined hazelnut oil (Sample 4) ozonated at a 7-8 flow rate for 60 minutes and obtained by hexane extraction. A) Ozonated nut oil (4) incubation after 48 hours; B) Ozonated nut oil (4) incubation after 72 hours.

7-8 and obtained by hexane extraction) had any toxic effect on HUVEC, H1299, and A549 cell lines.

It was found that even high concentrations (100 µg/mL) of all samples did not have any negative effects on cell viability in the healthy HUVEC line. However, it was found that Sample 5 (unrefined hazelnut oil ozonated at a 7-8 flow rate for 120 minutes and obtained by hexane extraction) at a concentration of 6.25 µg/mL reduced viability to 79% in the cancer cell line (H1299). It was also found that Sample 3 (unrefined hazelnut oil ozonated at a 7-8 flow rate for 120 minutes) at a concentration of 6.25 µg/mL reduced cell viability in the other cancer cell line, *i.e.* A549. It was determined that these newly synthesized ozonated hazelnut oils had effects on cell reproduction depending to the substance used for extracting oil or the duration of ozonization, and that they did not have any toxic effects on the healthy HUVEC line. In light of this information, it was concluded that hazelnut oil can be safely used after treatment with ozone without having any toxic effects and that it can be produced as a functional oil providing both the pharmacological and cosmetic effects of ozone.

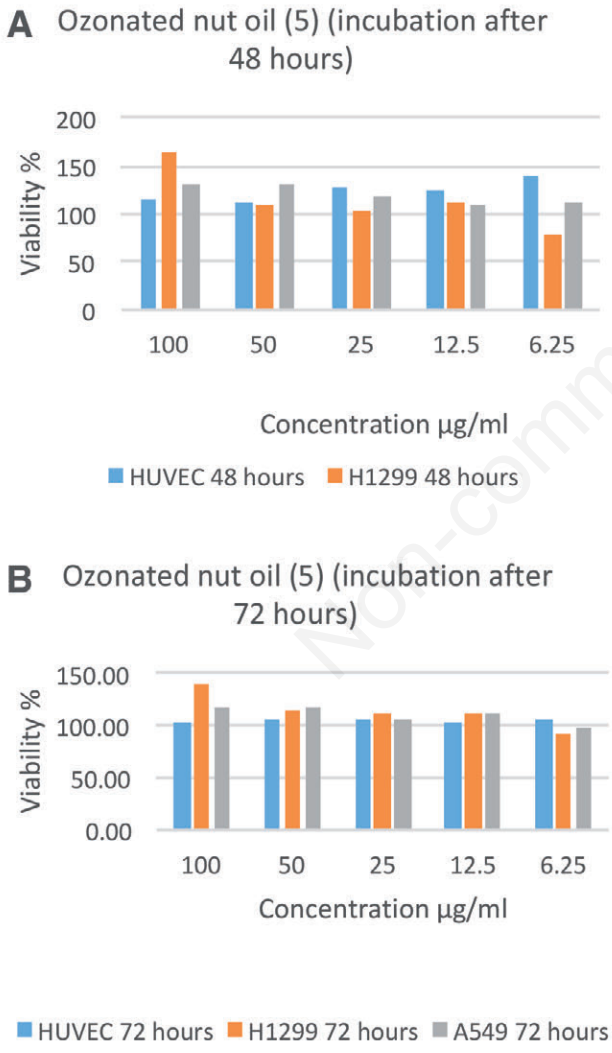

Figure 5. Viability rates of the cell lines after 48-72 hours of incubation of the unrefined hazelnut oil (Sample 5) ozonated at a 7-8 flow rate for 120 minutes and obtained by hexane extraction. A) Ozonated nut oil (5) incubation after 48 hours; B) Ozonated nut oil (5) incubation after 72 hours.

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