

# Investigation of Neuroprotective and Immunological Effects of Alpha Humulene *In Vitro* Co-Culture Model on SH-SY5Y Cell Line

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## Perihan *et al.*: Neuroprotective and Immunological Actions of Alpha-Humulene

Alpha humulene, the major active ingredient of the hops plant (*Humulus lupulus* L.), is a monocyclic sesquiterpene composed of three isoprene units containing three unpaired C=C double bonds. Beside hops plant, it is also commonly found in herbs such as cannabis, sage, basil, and ginseng. Alpha humulene is also known as alpha Caryophyllene and it has many effects such as anti-inflammatory, antimicrobial, antioxidant, anticancer and local anaesthetic. In this study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test was used to determine the concentration range of the cytotoxic effects of Alpha humulene in the SH-SY5Y cell line, a bone marrow derived human neuroblastoma cell line. In addition, the neuroprotective effects of Alpha humulene in the model of neurotoxicity induced by hydrogen peroxide, the half-maximal inhibitory concentration value of Alpha humulene on undifferentiated and retinoic acid induced differentiated SH-SY5Y was determined 221 µg/ml and >400 µg/ml cells using the Real-time cell analysis system. Apoptotic effect levels were determined by Acridine orange/Parkinson's disease, staining method. The SH-SY5Y cell line and the lipopolysaccharide stimulated Tohoku hospital pediatrics-1 cell line were incubated together to establish an *in vitro* co-culture model. In the *in vitro* co-culture model established, fluorescence measurements were taken in Cytation 3 multi-mode reader to determine the immunological effects of alpha humulene using lipopolysaccharide stimulation and multiple independent images were taken. The apoptotic effect caused by cellular damage caused by hydrogen peroxide alpha humulene was reduced.

**Key words:** Alpha humulene, neuroprotective effect, co-culture model, real-time cell analysis system dual purpose

Cannabinoids are metabolites comprising a group of chemical compounds that act through cannabinoid receptors found in essential oils of species belonging to the Cannabaceae family, such as *Eugenia caryophyllus*, *Humulus lupulus*, *Teucrium marum* and *Lantana achyranthifolia*. Cannabinoids work together with terpenes, which are secondary metabolites. Caryophyllenes are sesquiterpenes containing beta caryophyllene, alpha caryophyllene (Alpha humulene), and isocaryophyllene. Alpha humulene *Humulus lupulus* L. is a monocyclic sesquiterpene composed of three isoprene units found in the essential oils of the hop plant<sup>[1]</sup>. Alpha humulene has anti-inflammatory, antimicrobial, anticancer, antioxidant and local anesthetic effects<sup>[2]</sup>.

Along with the evolutionary process, there is an increase in the incidence of neurodegenerative diseases with the prolongation of human life. Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD), Amyotrophic Lateral Sclerosis (ALS), and Spinocerebellar Ataxia Disease (SCA) are common neurodegenerative diseases<sup>[3]</sup>. Unfortunately, there is no effective treatment for these diseases, even as a result of scientific studies carried out with the developing technology.

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Oxygen (O<sub>2</sub>), which is important for the vital activities of organisms is used in mitochondrial redox reactions and as a result of these reactions, reactive oxygen derivatives are formed. In case of excessive Reactive Oxygen Species (ROS) production, oxidative stress occurs. Oxidative stress can cause macromolecular damage, leading to atherosclerosis, diabetes, cancer, neurodegenerative diseases and aging.

It is accepted in the literature that cannabinoids are a useful therapeutic agent due to their low toxicity and are suitable for use in the treatment of neurological diseases<sup>[4]</sup>. In the Central Nervous System (CNS) of rodents and humans; the location of the CB1 receptor in the hippocampus, amygdala, hypothalamus, cerebellum, brain stem and spinal cord was determined<sup>[5]</sup>. Cannabinoids can reduce oxidative damage by acting as scavengers of ROS<sup>[6]</sup>. Cannabinoids exert an effect on macrophage and microglial cells by modulating Interleukin (IL)-1, Tumor Necrosis Factor-Alpha (TNF- $\alpha$ ) and IL-6 in neurodegeneration and neuroinflammatory states<sup>[7]</sup>. Cannabinoids have neuroprotective, anti-oxidative and anti-apoptotic effects against A $\beta$ .

In order to investigate the antiproliferative and antiapoptotic effects of Alpha humulene within the scope of the study, first of all, toxic/non-toxic concentrations were determined on SH-SY5Y cells by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) method. After that Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induced neurotoxicity model was established on SH-SY5Y cells differentiated into neuronal phenotype and cell viability values of the neuroprotective effect of Alpha humulene were determined by both the MTT method and the xCELLigence Real-Time Cell Analysis System Dual Purpose (RTCA-DP). In addition to the neurotoxicity model, an *in vitro* co-culture model was established by co-incubating the SH-SY5Y cell line and the Lipopolysaccharide (LPS)-stimulated Tohoku Hospital Pediatrics-1 (THP-1) cell line.

In conclusion, the antiproliferative, neuroprotective and antiapoptotic effects of Alpha humulene on the SH-SY5Y cell line were determined within the scope of the study.

## MATERIALS AND METHOD

### Cell lines development:

Roswell Park Memorial Institute Medium 1640 (RPMI-1640) medium containing 1 % Pen-Strep,

0.1 % Pyromycin, 10 % Fetal Bovine Serum (FBS) and 0.0004 %-mercaptoethanol was used to grow the THP-1 cell line.

The SH-SY5Y cell line was developed in 10 % FBS, 1 % penicillin-streptomycin, 1 % amphotericin-B, 0.1 % puromycin, and Dulbecco's Modified Eagle's Medium (DMEM) medium. For neuronal differentiation of SH-SY5Y cells, all-trans- Retinoic Acid (RA) was added a day after plating at final concentration 10  $\mu$ M in DMEM with 1 % FBS and maintained for 5 d.

### Differentiation of the SH-SY5Y human neuroblastoma cell line into neuronal phenotype:

SH-SY5Y cells were grown in DMEM medium containing 10 % FBS, 1 % penicillin/streptomycin until they reached a density of 70 %-80 %, then incubated for 5 d in differentiation medium containing 1 % FBS, 1 % penicillin/streptomycin, and 10  $\mu$ M RA to differentiate. When SH-SY5Y human neuroblastoma cells are undifferentiated, they grow in clusters to express immature neuron markers and undergo rapid proliferation. When cells differentiate with RA, proliferation decreases, polarization and neurite formation begin.

### Counting cells:

After visualizing with an inverted microscope that SH-SY5Y neuroblastoma cells reached a density of 70 %-80 %, the medium in the 75 cm<sup>2</sup> flask was removed and washed with 5 ml of Phosphate Buffer Solution (PBS). After washing, PBS was removed and 1000  $\mu$ l trypsin- Ethylenediamine Tetraacetic Acid (EDTA) was added into the flask and left to incubate in a Carbon dioxide (CO<sub>2</sub>) oven for 5 min. After incubation, the cells were removed from the surface by applying mechanical force to the flask. 5 ml of fresh medium was added to the flask and the suspended cell group was transferred to a 50 ml flask. Centrifuged at 1250 rpm for 5 min, the cells settle to the bottom and thus the supernatant was removed. Pipetting was performed by adding 1 ml of suitable fresh medium (DMEM) to the cell pellet remaining in the falcon. Homogeneously distributed SH-SY5Y cells after pipetting were counted on Cedex (Roche - Germany) using Trypan Blue dye.

THP-1 cells developed and contained in flasks of 75 cm<sup>2</sup> were collected and transferred to 50 ml falcons and centrifuged at 1100 rpm for 5 min. After centrifugation, the supernatant was removed and 1

ml of fresh medium was added to the settled THP-1 cells and pipetting was performed. THP-1 cells showing a homogeneous distribution after pipetting were counted on Cedex (Roche-Germany) using Trypan Blue.

#### **Cytotoxicity test (MTT test):**

MTT test was performed both RA-differentiated and undifferentiated SH-SY5Y cells. After the cells were counted,  $5 \times 10^3$  for RA-differentiated SH-SY5Y and  $1 \times 10^4$  for undifferentiated SH-SY5Y cells were calculated in each well and 100  $\mu$ l was distributed to 96-well plates. The plates were incubated for 24 h in a 5 % CO<sub>2</sub> oven.

After 24 h of incubation, the media on the plates were withdrawn and discarded. For Alpha humulene dilution was prepared in the range of 400  $\mu$ M-50  $\mu$ M and given to each well in 100  $\mu$ l medium. They were placed in the oven again with 5 % CO<sub>2</sub> and left 24 h for undifferentiated and 5 d RA-differentiated SH-SY5Y incubation.

At the end of the incubation, the media were withdrawn and discarded. MTT prepared at a ratio of 1:9 in the dark was distributed to each well as 10  $\mu$ l (5 mg/ml) and incubated in an oven with 5 % CO<sub>2</sub> for 4 h.

At the end of the incubation, the media on the plates were withdrawn and discarded. For H<sub>2</sub>O<sub>2</sub> dilution was prepared in the range of 400  $\mu$ M-1  $\mu$ M and given to each well in 100  $\mu$ l medium. They were placed in the oven again with 5 % CO<sub>2</sub> and left for 2 h incubation.

After 2 h of incubation, the media were withdrawn and discarded. MTT prepared at a ratio of 1:9 in the dark was distributed to each well as 10  $\mu$ l (5 mg/ml) and incubated in an oven with 5 % CO<sub>2</sub> for 4 h.

After 24 h of incubation, the media on the plates were withdrawn and discarded. For Alpha humulene dilution was prepared in the range of 400  $\mu$ M-50  $\mu$ M and given to each well in 100  $\mu$ l medium. They were placed in the oven again with 5 % CO<sub>2</sub> and left 24 h for undifferentiating and 5 d for RA-differentiation SH-SY5Y incubation. At the end of the incubation, the existing medium was changed with the medium containing the non-toxic concentration of H<sub>2</sub>O<sub>2</sub>, which was also determined by the MTT method, and it was left to incubate for 2 h.

After 2 h of incubation, the media were withdrawn and discarded. MTT prepared at a ratio of 1:9 in the dark was distributed to each well as 10  $\mu$ l (5 mg/ml)

and incubated in an oven with 5 % CO<sub>2</sub> for 4 h.

After the cells were counted,  $5 \times 10^3$  for RA-differentiated SH-SY5Y cells were calculated in each well and 100  $\mu$ l was distributed to 96-well plates. The plates were incubated for 5 d in a 5 % CO<sub>2</sub> oven. On the 3<sup>rd</sup> d, the medium was replaced with fresh medium. After the THP-1 cells reaching the appropriate density were counted, they were inoculated into 6-well plates at  $2 \times 10^5$  cells per well. While *Escherichia coli* (*E. coli*) LPS was added to one well of suspended THP-1 cells at a rate of 1  $\mu$ g/ml and the other well at a rate of 100 ng/ml, 1 well was separated as a control group and left to incubate for 4 h. At the end of the incubation, the cell-medium in each well was collected in the same falcon and centrifuged at 1100 rpm for 5 min. At the end of centrifugation, concentrations of 400-50  $\mu$ g/ml prepared from the stock solution of alpha humulene were applied on SH-SY5Y cells in the obtained supernatant. At the end of the incubation period, the non-toxic concentration of H<sub>2</sub>O<sub>2</sub> was added to the medium and left for 2 h incubation. At the end of the incubation, MTT method was applied.

MTT dye was drawn off from SH-SY5Y cell line whose incubation period had expired. Later, 100  $\mu$ l of Dimethyl Sulfoxide (DMSO) was given to each well and measured at 540 nm absorbance in an Enzyme-Linked Immunoassay (ELISA) device within half an hour.

Cell viability was calculated from the obtained data and plotted on GraphPad, and significant results were selected for SH-SY5Y cell line and tested on xCELLigence device.

#### **Real-time cell analysis system (xCELLigence Technology):**

For the SH-SY5Y cell line; 100  $\mu$ l of clean DMEM medium was added to the special E-plates used for the xCELLigence instrument. The xCELLigence device was stopped to plant cells on the clean media that were taught, and after counting the cells in the Cedex device, 100  $\mu$ l was distributed to each well except one well by calculating  $1 \times 10^5$  for undifferentiated cells,  $5 \times 10^3$  for RA-differentiated per well. Medium control was performed by adding 100  $\mu$ l of clean medium to the empty well. The cells, which were kept in the sterile cabinet for 15 min, were placed in the xCELLigence device and the program continued to be run from where it left off. When the number of cells measured with a clean medium for 24 h

reached the highest point, the device was stopped and E-plaques were removed. 100  $\mu$ l was withdrawn from the medium and the concentrations prepared in the range of 400  $\mu$ l, 200  $\mu$ M - 100  $\mu$ M and 50  $\mu$ M for Alpha humulene were given. The device was restarted and measurements were taken for 24 and 48 h.

### Apoptosis Test (Acridine Orange (AO)-Propidium Iodide (PI) double staining):

AO/PI double staining was performed for RA-differentiated and undifferentiated SH-SY5Y cells. After the cells were counted,  $2 \times 10^5$  for RA-differentiated SH-SY5Y and  $5 \times 10^5$  for undifferentiated SH-SY5Y cells were calculated in each well and 1 ml was distributed to 6-well plates. 24 h for undifferentiated cells, 5 d for RA-differentiation left for incubation. At the end of the incubation, the cells were counted,  $2 \times 10^5$  THP-1 cells were calculated in each well and 1 ml was distributed to 6-well plates. 100 ng/ml of E.coli LPS was added to each well of the suspended THP-1 cells and incubated for 4 h. At the end of the incubation, the cell-medium in each well was collected in the same falcon and centrifuged at 1100 rpm for 5 min. A co-culture model was established with the SH-SY5Y cell line RA-differentiated into neuronal phenotype and THP-1 cells stimulated with E.coli LPS. Alpha humulene dilution was prepared in the range of 400  $\mu$ M-50  $\mu$ M and given to each well in 1 ml medium.

The plates were incubated for 24 h in a 5 % CO<sub>2</sub> oven. In order to determine apoptosis with AO/PI staining after 24 h, it was incubated for 2 h with H<sub>2</sub>O<sub>2</sub> IC<sub>50</sub> value and staining procedure was applied at the end of the incubation period.

At the end of the applied procedure, fluorescence measurements at a wavelength of 525 excitation and 617 nm emission were taken, and multiple independent images were taken in the Cytation 3 multi-mode reader.

## RESULTS AND DISCUSSION

MTT results for Alpha humulene of SH-SY5Y cells were read in Cytation 3 cell imaging multi-mode reader at 540 nm wavelength, 8 replicates for each concentration. The average of the absorbance values of the control group was accepted as 100 % and the % viability values of other concentrations were calculated. Graphs generated from the data that obtained as a result of 24 h incubation of undifferentiated SH-SY5Y cells and RA-differentiated SH-SY5Y cells are shown in fig. 1 and fig. 2.

The graph of the neuroprotective effect of Alpha humulene, formed from the data obtained as a result of 24 h incubation in a co-culture model created with SH-SY5Y cell line RA-differentiated into neuronal phenotype and stimulated with *E. coli* LPS, is shown in fig. 3.

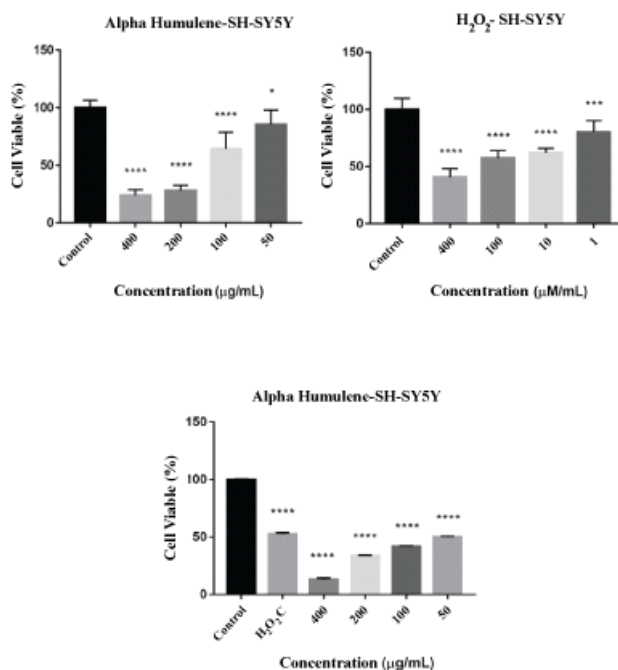


Fig. 1: Cell viability values and statistical evaluation of Alpha humulene and H<sub>2</sub>O<sub>2</sub> concentrations on SH-SY5Y cell line (\*\*\*\*p<0.0001, n=8)

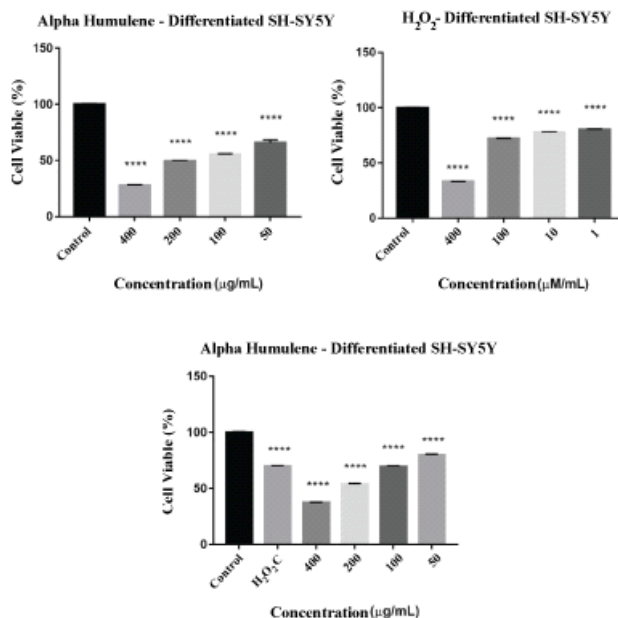


Fig. 2: Cell viability values and statistical evaluation of Alpha humulene and H<sub>2</sub>O<sub>2</sub> concentrations on RA-differentiated SH-SY5Y cell line (\*\*\*\*p<0.0001, n=8)

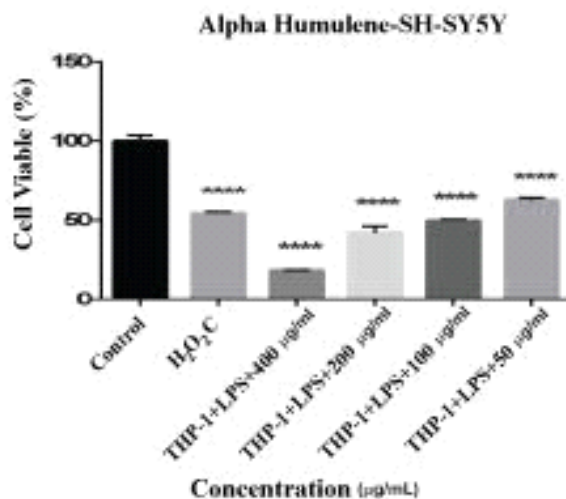


Fig. 3: Cell viability values and statistical evaluation of Alpha humulene and H<sub>2</sub>O<sub>2</sub> concentrations in the co-culture model

Determination of IC<sub>50</sub> Value of Alpha humulene and H<sub>2</sub>O<sub>2</sub> for undifferentiated and RA-differentiated SH-SY5Y Using xCELLigence real-time cell analysis system

As a result of the screenings performed with the MTT cytotoxicity test, the concentration ranges of Alpha humulene (400-50 µM), H<sub>2</sub>O<sub>2</sub> (400-1 µM) cell line was determined and applied in the RTCA DP system. The device was stopped 48 h after the application of the concentrations (72 h in total) and the IC<sub>50</sub> values were automatically calculated by the RTCA DP Software 1.2.1 program. In SH-SY5Y cells, IC<sub>50</sub> values at 24 and 48 h calculated according to the

real-time cell index data of H<sub>2</sub>O<sub>2</sub> were determined as 42 µM and 38 µM, respectively and are shown in fig. 4 and fig. 5.

IC<sub>50</sub> values were calculated in the RTCA-DP system with 50, 100, 200 and 400 µg/mL Alpha humulene on SH-SY5Y cells and H<sub>2</sub>O<sub>2</sub> concentrations with an IC<sub>50</sub> value of 42 µM according to the slope graph. The calculated IC<sub>50</sub> values are 221 µg/mL at 24 h and 209 µg/ml at 48 h. The 24 h cell proliferation curves of the concentrations of 400-50 µg/ml Alpha humulene and 42 µM H<sub>2</sub>O<sub>2</sub> in SH-SY5Y cells with the RTCA-DP analysis system are shown in fig. 6.

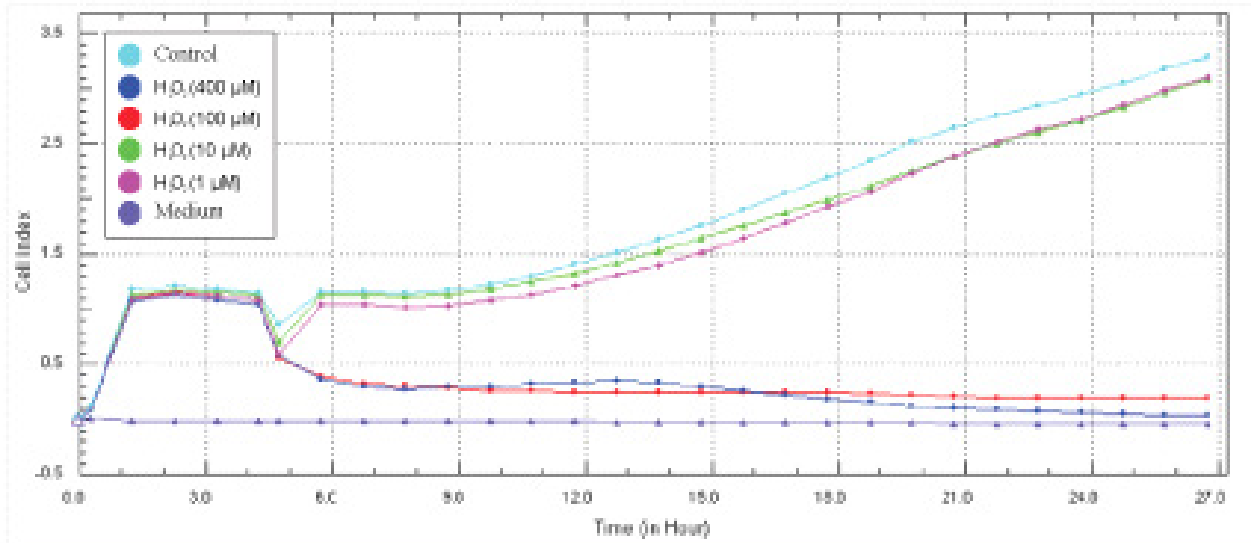


Fig. 4: SH-SY5Y cell proliferation curve of 24 h concentrations of H<sub>2</sub>O<sub>2</sub>

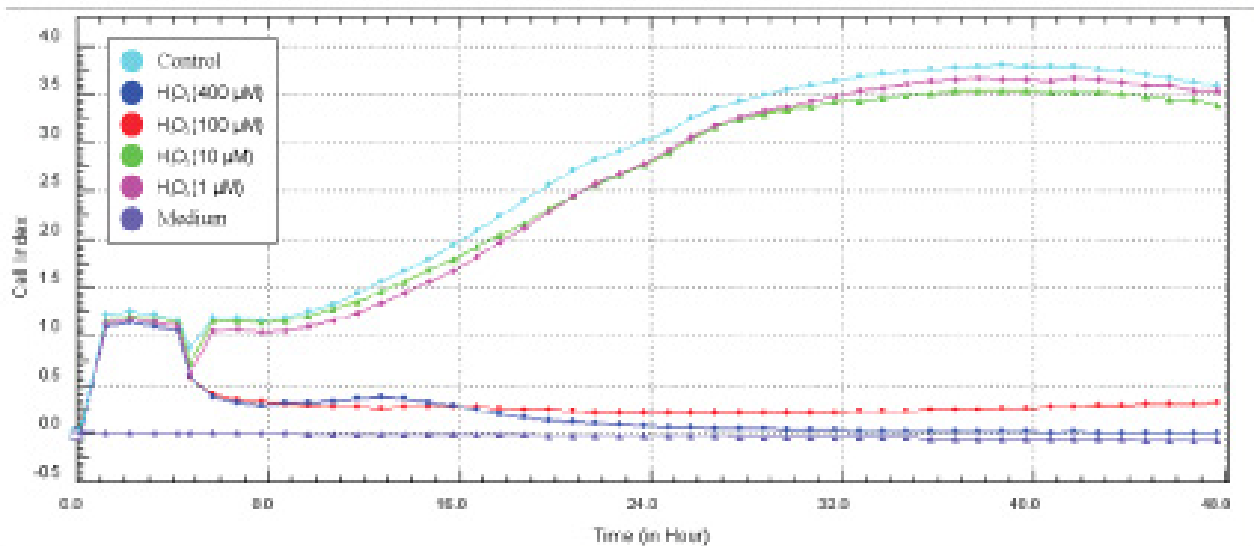


Fig. 5: SH-SY5Y cell proliferation curve of 48 h concentrations of H<sub>2</sub>O<sub>2</sub>

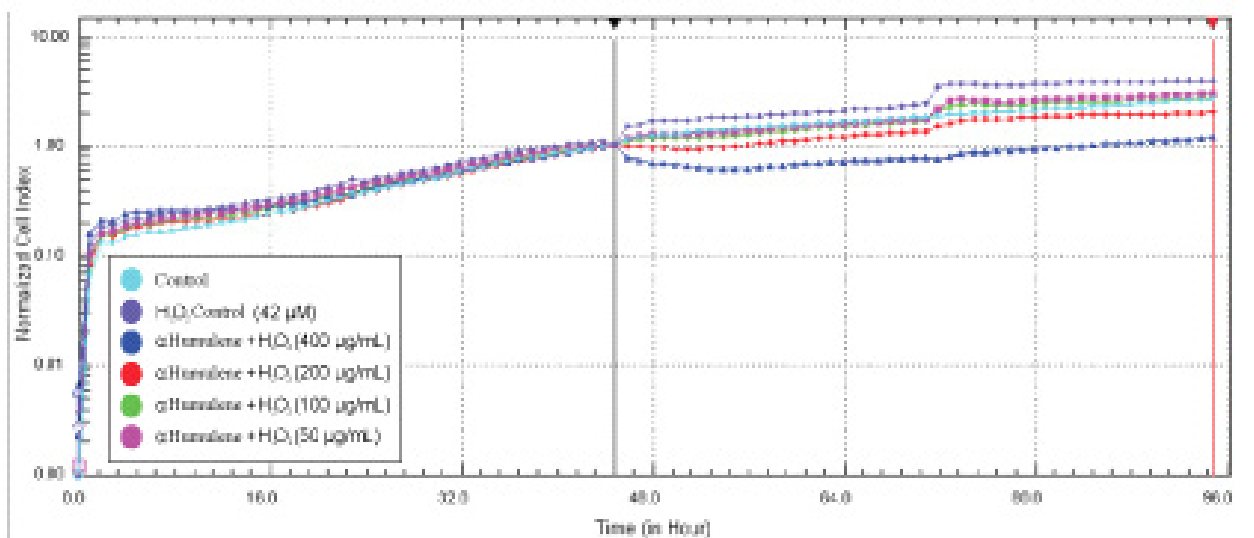


Fig. 6: SH-SY5Y cell proliferation curve of 24 h concentrations of Alpha humulene and H<sub>2</sub>O<sub>2</sub>

When its neuroprotective effects on RA-differentiated SH-SY5Y cells are evaluated in real time, the neuroprotective effect of Alpha humulene on RA-differentiated SH-SY5Y cells is  $>400 \mu\text{g/ml}$  at 48 h calculated based on real-time cell index data. The 48 h cell proliferation curves of the concentrations of 50, 100, 200 and 400  $\mu\text{g/ml}$  Alpha humulene and 42  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  in RA-differentiated SH-SY5Y cells with the RTCA-DP analysis system are shown in fig. 7.

First of all, concentration scanning was performed for Alpha humulene and  $\text{H}_2\text{O}_2$  in high concentration ranges by MTT study and cytotoxicity analysis was performed in the xCELLigence system and  $\text{IC}_{50}$  value was determined according to 24 h cell index values. With the obtained  $\text{IC}_{50}$  values, the non-cytotoxic

concentration to be used in the next experiments was determined.

Fluorescence microscopy was used to observe the morphological changes and apoptotic properties of undifferentiated and RA-differentiated SH-SY5Y cells that underwent the AO/PI staining procedure. AO stains cell nuclei bright green to indicate viable cells, while PI stains nuclei red to indicate damaged and necrotic cells<sup>[8]</sup>. Control cells that were not treated with Alpha humulene and  $\text{H}_2\text{O}_2$  were observed to have bright green and intact nuclei. In  $\text{H}_2\text{O}_2$  control cells treated with 42  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  for 2 h, green nuclei indicate viable cells, while those stained red indicate apoptosis in  $\text{H}_2\text{O}_2$  induced cytotoxicity as shown in fig. 8 and fig. 9.

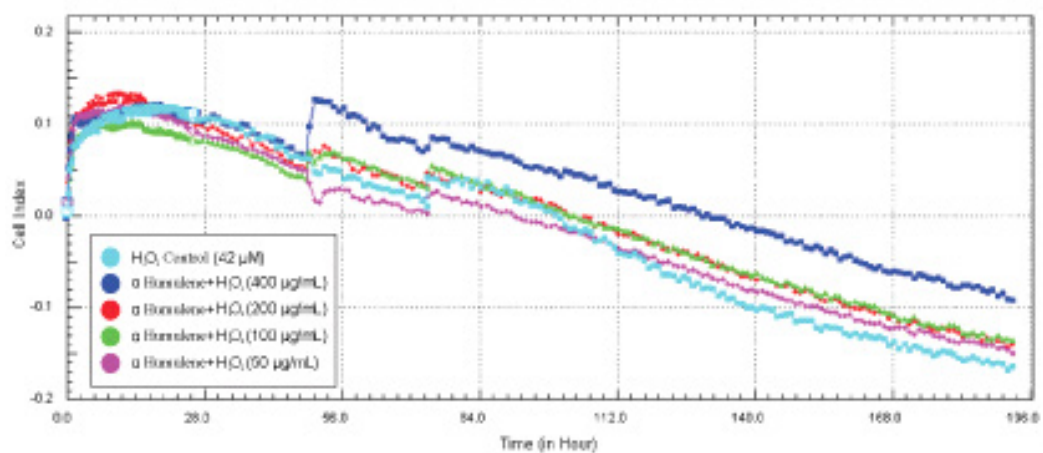


Fig. 7: RA-differentiated SH-SY5Y cell proliferation curve of 48 h concentrations of alpha humulene and  $\text{H}_2\text{O}_2$

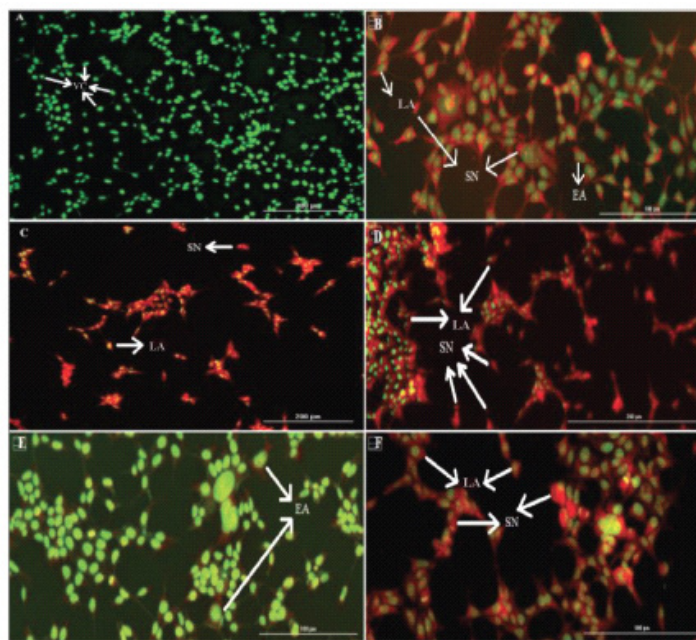
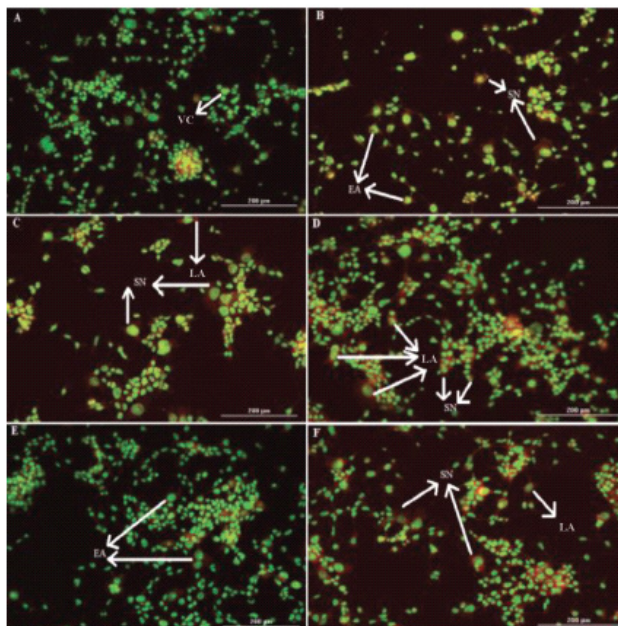


Fig. 8: AO-PI double staining cell morphological assessment in co-culture: model (10X). (A): Control; (B):  $\text{H}_2\text{O}_2$  control; (C): 400  $\mu\text{g/ml}$ ; (D): 200  $\mu\text{g/ml}$ ; (E): 100  $\mu\text{g/ml}$  and (F): 50  $\mu\text{g/ml}$  VC: Viable cell, LA: Late apoptosis SN: Secondary necrosis and EA: Early apoptosis



**Fig. 9: AO-PI double staining cell morphological assessment**

After cell treatment with alpha humulene at a concentration of 400  $\mu\text{g/ml}$ , necrosis cells were visualized in red. When the cells were treated with 200  $\mu\text{g/ml}$  concentration of alpha humulene, it was determined that the cells in orange color increased significantly and accordingly, it caused late apoptosis. Early apoptosis features including chromatin condensation were observed after cell treatment with 100  $\mu\text{g/ml}$  of Alpha humulene. Alpha humulene was found to increase apoptosis with red-orange colored cells after cell treatment with a concentration of 50  $\mu\text{g/ml}$ . It was determined that the cells that were not treated with Alpha humulene and  $\text{H}_2\text{O}_2$  had normally show round and green nuclei. Membrane bubbling and chromatin condensation were detected in SH-SY5Y cells treated with 100  $\mu\text{g/ml}$  of Alpha humulene for 24 h and 42  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 2 h, which are signs of early apoptosis.

Presence of reddish-orange color was detected in cells treated with the highest and lowest concentrations of alpha humulene (400 and 200 and 50  $\mu\text{g/ml}$ ), which was determined as the late stage of apoptosis or necrosis.

Neurodegenerative disorders are characterized by many diseases with macro and micro changes occurring in different parts of the brain<sup>[9]</sup>. AD, PD, HD, ALS, and SAD are common neurodegenerative diseases. AD is a neurodegenerative disease characterized by the gradual loss of behaviour, memory, cognition and functionality, which significantly reduces the

quality of daily life<sup>[10]</sup>. Although age is considered to be the primary risk factor in AD, the pathology of the disease originates from free radicals<sup>[11]</sup>. The histopathological changes in AD result from the accumulation of extracellular Amyloid Beta ( $\text{A}\beta$ ) plaques and intracellular tau Neurofibrillary Tangles (NFY), and its pathology is thought to be associated with oxidative stress together with the neurotoxicity of  $\text{H}_2\text{O}_2$  and  $\text{A}\beta$  peptide<sup>[12]</sup>. PD, which is the second most common neurodegenerative disease, is characterized by the loss of dopaminergic neurons in the pars compacta part of the substantia nigra of the brain<sup>[13]</sup>. Pathological mechanisms resulting from degeneration of ROS and dopaminergic neurons are due to excessive production of ROS or other free radicals in the brain, mitochondrial dysfunction or inflammation. It has been determined that the increase in ROS causes oxidative stress and ROS plays an important role in the development of PH by causing  $\alpha$ -synuclein accumulation and proteasomal degradation<sup>[14-16]</sup>. HD, another neurodegenerative disease, is an inherited neurodegenerative disease that occurs as a result of mitochondrial dysfunction and neurodegeneration with ROS-induced oxidative stress and apoptosis. HD is characterized by involuntary movements, cognitive impairment, neuropsychiatric symptoms, and premature death<sup>[17-19]</sup>. ALS, another neurodegenerative disease, is a disease that progresses with mitochondrial dysfunction with increased production of ROS, characterized by loss of motor neurons<sup>[20,21]</sup>.

It has been accepted in the literature that cannabinoids are a useful therapeutic agent due to their low toxicity and suitable for use in the treatment of neurological diseases<sup>[4,22]</sup>. In this study, we investigated the antiproliferative, antiapoptotic and neuroprotective effects of alpha humulene, the characteristic terpene of *Humulus lupulus*, in an *in vitro* co-culture model.

As a result of the literature review, although similar studies were found in human neuroblastoma cell lines SH-SY5Y and THP-1 monocyte cell lines, *in vitro* co-culture studies with Alpha humulene were not found.

First of all, the MTT assay was used to investigate the cytotoxic effects of alpha humulene on SH-SY5Y cells. MTT assay was performed to determine the concentration range for SH-SY5Y cells. We determined the IC<sub>50</sub> values obtained from the cytotoxicity results using the xCELLigence real-time cell analysis system RTCA-DP. In the xCELLigence real-time cell analysis system, SH-SY5Y cells were RA-differentiated in the instrument for 5 d. After being treated with concentration groups at the end of the differentiation process, cell damage was performed with H<sub>2</sub>O<sub>2</sub> before the experiment.

According to the MTT results we obtained, significant results were obtained in the range of 400-50 µg/ml of Alpha humulene in cell damage induced by H<sub>2</sub>O<sub>2</sub> on SH-SY5Y cells. H<sub>2</sub>O<sub>2</sub> was studied in the range of 400-1 µM in RTCA-DP. With H<sub>2</sub>O<sub>2</sub> induced cell damage, H<sub>2</sub>O<sub>2</sub> was studied in the concentration of 42 µM and Alpha humulene in the range of 400-50 µg/ml. The results obtained with RTCA-DP were calculated by the RTCA-DP Software 1.2.1 program. The IC<sub>50</sub> for H<sub>2</sub>O<sub>2</sub> on the SH-SY5Y cell line is 42 µM at 24 h and 38 µM at 48 h. The IC<sub>50</sub> of Alpha humulene for the neuroprotective effect on the undifferentiated SH-SY5Y cell line is 221 µg/ml at 24 h and 209 µg/ml at 48 h. The IC<sub>50</sub> of Alpha humulene for neuroprotective effect on the SH-SY5Y cell line RA-differentiated into neuronal phenotype was calculated as >400 µg/ml.

Our study is in parallel with a study that investigated the cytotoxic effect of Alpha humulene on various human tumor cell lines (MCF-7, DLD-1, L-929) by MTT method. According to the results obtained, the IC<sub>50</sub> value of alpha humulene for the MCF-7 cell line was 10 mg/ml, the IC<sub>50</sub> value for the DLD1 cell line was >40 µg/ml, and the IC<sub>50</sub> value for the L-929 cell line was >40 µg/ml<sup>[2]</sup>.

In the literature, there are neuroprotective effect studies of microglia activated by an inducer such as LPS, in which an *in vitro* co-culture model was established with the SH-SY5Y human neuroblastoma cell line<sup>[23-25]</sup>.

We also evaluated the apoptotic effects of Alpha humulene, whose IC<sub>50</sub> values we found in our study, on SH-SY5Y cells. In this context, independent images were taken using Cytation 3 Multi-Mode Reader to determine the apoptosis for neuroprotective effect in the co-culture model created with SH-SY5Y cell line RA-differentiated by AO/PI staining method and THP-1 cells stimulated with *E. coli* LPS.

In one of the previous studies, the neuroprotective effect of ethyl acetate extracts of germinated brown rice on H<sub>2</sub>O<sub>2</sub> induced cell damage on the SH-SY5Y cell line was investigated. The anti-apoptotic effects of the extracts were evaluated by AO/PI staining method at 1-30 ppm concentrations and 250 µM H<sub>2</sub>O<sub>2</sub>. As a result, it was reported that ethyl acetate extracts of germinated brown rice increased cell viability, showed anti-apoptotic effect of cells, and cells tended to less apoptosis<sup>[8]</sup>.

Lastly, according to the results obtained from fluorescence measurements, H<sub>2</sub>O<sub>2</sub> IC<sub>50</sub> 42 µM, Alpha humulene 50, 100, 200, 400 µg/ml+42 µM of H<sub>2</sub>O<sub>2</sub> (p<0.0001\*\*\*\*) concentration groups were found to be significant. In one of the previous studies, the cytotoxic effect of 7-Geranyloxycinnamic at concentrations of 1.04-16.67 µM was examined, 5.97 µM of curcumin and 300 µM of H<sub>2</sub>O<sub>2</sub> were incubated for 4 h. According to the results obtained by applying the AO/PI staining procedure, they reported that the protective effect of 7-Geranyloxycinnamic and curcumin may be related to its ability to prevent apoptosis<sup>[26]</sup>.

As a result, in the light of the information obtained from the literature review for this study, it was determined that Alpha humulene, which has antimicrobial, anticancer and local anesthetic properties, has neuroprotective properties against H<sub>2</sub>O<sub>2</sub> induced cell damage. In the *in vitro* co-culture model created with THP-1 cells stimulated with *E. coli* LPS, the apoptotic stages of the effects of Alpha humulene occurring with different concentration ranges were morphologically observed.

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### Conflict of interests:

The authors declared no conflict of interests.

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