EURASIAN JOURNAL of MOLECULAR & BIOCHEMICAL SCIENCES

(EURASIAN MOL BIOCHEM SCI)

e-ISSN: 2822-4019

Volume : 1 - Number: 2 - September/October 2022

https://dergipark.org.tr/tr/pub/ejmbs
https://dergi.erzurum.edu.tr/ejmbs

Eurasian Journal of Molecular and Biochemical Sciences

Official Journal of Erzurum Technical University Faculty of Science

All articles in this journal are available from https://dergi.erzurum.edu.tr/ejmbs

Eurasian Mol Biochem Sci is published twice a year (March/April and September/October).

Indexing

Eurasian Journal of Molecular and Biochemical Sciences (Eurasian Mol Biochem Sci) is indexed by *Crossref, Index Copernicus, Google Scholar*.

Eurasian Journal of Molecular and Biochemical Sciences

Official Journal of Erzurum Technical University Faculty of Science

Year	Volume	Issue / Number
2022	2	1

Eurasian Journal of Molecular and Biochemical Sciences

Official Journal of Erzurum Technical University Faculty of Science

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Comparison of Iflavirus Diversity in Agricultural and Forest Pest Lepidoptera

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Cite: *Eroğlu GB. Comparison of Iflavirus Diversity in Agricultural and Forest Pest Lepidoptera. Eurasian Mol Biochem Sci 2022;1(2): 1-5.*

Received: 22 May 2022, Accepted: 12 September 2022 *DOI. 10.54672/ejmbs.2022.7*

Abstract

Iflaviridae is a novel and important family of viruses in the *Picornavirales* order that infects invertebrates. Iflaviruses are non-enveloped, linear, positive single-stranded RNA viruses. The importance of iflaviruses in agriculture and forestry is twofold because these viruses, both cause economic loss by making insects such as honey bees and silkworms ill and prevent economic loss by making agricultural and forest pest insects ill. Therefore, iflaviruses contain highly interesting isolates that infect both beneficial and harmful insects in agriculture. Its genome structure, like most RNA viruses, has a very small genome size (8.8-10 kb), with a single open reading frame in the entire genome. The genomes of 14 infective iflaviruses that infect agricultural and forest pest insects from different countries have been analyzed so far. In this study, the similar relationship between these 14 viruses, whose complete genomes are available, was analyzed according to the complete sequence of the polyprotein. The results showed that the virus isolates obtained from forest pest insects were closely related to each other. Similarly, it was revealed that the iflaviruses obtained from insects that damage agricultural products are more similar to each other. In addition, the results from this study support previous studies on adding a new genus to the *Iflaviridae* family, which has only one genus.

Keywords: Iflaviruses, pest insects, phylogeny, Kimura-2 parameter

Introduction

The *Iflaviridae* is a new virus family established about 10 years ago (Carstens & Ball, 2009). It is in the Picornavirales order and consists of a single genus (*Iflavirus*). The family name is derived from the infectious flacherie virus, the type species of the genus *Iflavirus* (1). Iflaviruses have spherical virions and a non-enveloped icosahedral structure with a diameter of approximately 22-30 nm (1-3). Members of the Iflaviridae family cause infection in many arthropods, especially in Lepidoptera, Hymenoptera, and Hemiptera orders (4). Therefore, iflaviruses are evaluated from two perspectives. The first is to protect beneficial insects from this viral disease by biologically controlling isolates of the virus that cause disease in honey bees (5-7). Because iflaviruses spread rapidly in

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these insects, they cause deformation, behavioral changes, and death in individuals. These viruses include deformed wing virus (DWV) and sacbrood virus (SBV). So far, there are many studies on DWV and SBV but the knowledge on iflaviruses in general is limited due to the fact that the iflaviruses are found in agricultural and forest pest insects generally asymptomatic (8-12). Infection of agricultural and forest pest insects with the virus is both by horizontal and vertical transmissions (13).

Most of the agricultural and forest pest insects are in the order Lepidoptera (14, 15). Iflaviruses cause infection in these insects alone and or coinfection with other insect viruses (16). Iflaviruses generally reproduce asymptomatically in insect larvae of agricultural pests (17). In recent years, with the development of omic technologies, many new infective viruses have been identified from larvae of agricultural and forest pests and genome analysis of many of them has been performed (16,18-24). Genome analysis studies provide valuable information for the evaluation of the relationships between viral isolates in the same family. In this study, the relationships between 14 isolates of iflavirus isolated from agricultural and forest pests, whose whole genome sequences are available on the NCBI database were examined.

Materials and Methods

Kimura-2 Parameter Analysis: To evaluate the relationship between iflaviruses isolated from agricultural and forest pests, those with complete genome sequences available in the NCBI database were used (Table 1). The transition/transversion ratios in the polyproteins of these isolates were determined by the Kimura analysis and the close relationship between them was evaluated. This analysis considered the complete ORF coding for the polyprotein of 14 iflaviruses. Sequences were aligned with the BioEdit program and the distance analysis (Kimura-2 parameter) in the MEGA 11 program was performed.

Phylogeny: The amino acid sequences encoded by the complete polyprotein of the 14 iflavirus isolates were aligned using the program BioEdit (7.1.3.0). For phylogenetic tree analysis, the Jones-Taylor-Thornton (JTT) model with 1000 bootstrap in the Maximum Likelihood method was used to generate a phylogenetic tree using the MEGA 11 program.

Results

Kimura-2 parameter analysis: The transition/transversion distances between the isolates were investigated according to the complete nucleotide sequence of the polyprotein ORF among the isolates that have been detected so far in agricultural and forest pest insects and whose whole genome sequence has been analyzed (Table 2). According to the results obtained, the nucleotide distance between the iflavirus isolates (except Opsiphones invirae iflavirus) obtained from insect species that cause damage in the forest area was below 0.5. The distance between Lymantria dispar iflavirus Russia1 and Russia2 isolates was 0.002 while the distance between Lymantria dispar iflavirus Russia isolates and Lymantria dispar iflavirus USA isolate was 0.055. It was found that the nucleotide distance of the iflavirus Turkey isolates of *Thaumetopoea pityocampa*, a very important forest pest, with other forest pests was between 0.347-0.369. While the distance between the iflavirus isolates of *Antheraea mylitta* and *Antheraea pernyi* in the same genus is 0.287, their distance from other forest pests is between 0.349-0.374. However, it is seen that Helicoverpa armigera iflavirus isolate which is an important agricultural pest, is closer to the iflavirus isolate obtained from forest pests (0.429-0.466) rather than iflaviruses isolated from other agricultural pests (1.254-1.631). There is an interesting situation among the three viruses isolated from *Spodoptera exigua*. Spodoptera exigua iflavirus Spain1 isolate has a value (1.884-1.885) that is quite far from Spain2 and Korea isolates. The nucleotide distance from all isolates

Table 1. Information of the Iflavirus genomes used in the analyzes

Host name	Host family	Pest plant	Origin	Genome size	Accession number	References
Antheraea pernyi	Saturniidae	Forest	China	10176 kb	NC_023483.1	Geng et al., 2014
Antheraea mylitta		Forest	India	9728 kb	MW115117.2	unpublished
Plutella xylostella	Plutellidae	Cabbage	Australia	9623 kb	MN328434.1	unpublished
Plutella xylostella			China	9580 kb	KY435608.1	unpublished
Helicoverpa armigera	Noctuidae	Variety agricultural products	China	10017 kb	NC_033619.1	Yuan et al., 2017
Heliconius erato	Nymphalidae	*Passiflora suberosa*	Costa Rica	9910 kb	NC_024016.1	Smith et al., 2014
Lymantria dispar	Erebidae	Forest	Russia1	10121 kb	MT753155.1	unpublished
Lymantria dispar			Russia2	9996 kb	MN938851.1	Pavlushin et al., 2021
Lymantria dispar			USA	10044 kb	NC_024497	Carrillo-Tripp et al., 2014
Opsiphones invirae	Nymphalidae	Forest	Brazil	9855 kb	NC_027917.1	Silva et al., 2015
Spodoptera exigua	Noctuidae	Variety agricultural products	Spain1	10347 kb	NC_016405.1	Millán-Leiva et al., 2012
Spodoptera exigua			Spain2	9504 kb	KJ186788.1	Jakubowska et al., 2014
Spodoptera exigua			Korea	9501 kb	NC_023676.1	Choi et al., 2012
Thaumetopoea pityocampa	Thaumetopoeidae	Forest	Turkey	9816 kb	KP217032.1	Jakubowska et al., 2014

except Opsiphones invirae iflavirus was also quite far (1.343-1.809). The only virus isolate closely related to Spodoptera exigua iflavirus Spain1 was Opsiphones invirae iflavirus isolate (0.676) (Table 2).

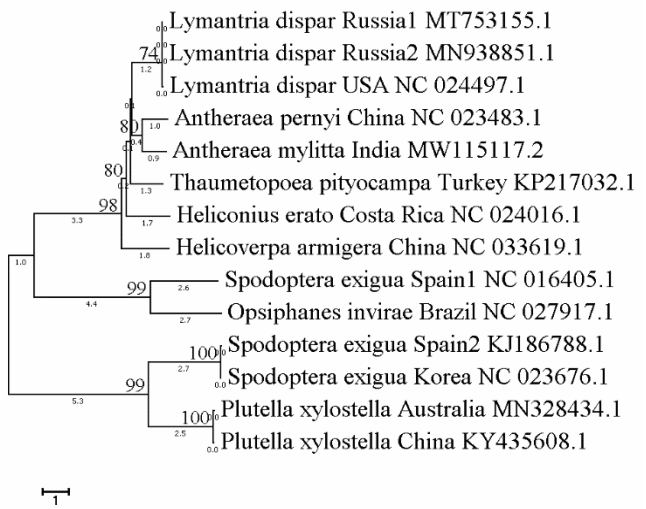

Figure 1. The Immunocytochemical illustration of anti-Beclin-1 and anti-LC-3 staining of HT-29 cells incubated with 0nM, 10nM, 20nM, and 40nM concentration of Bortezomib for 24h.

Phylogeny: As a result of the phylogenetic analysis of the 14 isolates based on their complete polyprotein amino acid sequences, it was seen that with some exceptions, insect viruses of forest pests clustered together while insect viruses of agricultural pests separately clustered together and the results supported the Kimura-2 parameter analysis. Exceptionally, it was observed that the iflavirus isolate from the agricultural pest *Helicoverpa armigera* clustered far away from the iflavirus isolates of other agricultural pests (*Plutella xylostella* and *Spodoptera exigua*), but clustered close to the iflaviruses isolated from forest pests. In addition, it was observed that Spodoptera exigua iflavirus Spain1 isolate clustered closely with Opsiphones invirae iflavirus isolate instead of other Spodoptera exigua iflavirus isolates (Figure 1).

Discussion

The family *Iflaviridae* is newly established and has only one genus (*Iflavirus*) (25). While infective viruses

Table 2. Kimura-2 parameter analysis of iflavirus genomes

Spodoptera exigua Spain1 NC016405.1													
Spodoptera exigua Spain2 KJ186788.1	1,884												
Spodoptera exigua Korea NC 023676.1	1,885	0,015											
Helicoverpa armigera China NC 033619.1	1,349	1,600	1,609										
Lymantria dispar Russia1 MT753155.1	1,350	1,574	1,583	0,433									
Lymantria dispar Russia2 MN938851.1	1,345	1,575	1,581	0,433	0,002								
Lymantria dispar USA NC024497.1	1,353	1,590	1,592	0,436	0,055	0,055							
Thaumetopoea pityocampa Turkey KP217032.1	1,368	1,592	1,588	0,453	0,348	0,347	0,350						
Heliconius erato Costa Rica NC024016.1	1,392	1,614	1,609	0,466	0,429	0,429	0,436	0,395					
Plutella xylostella Australia MN328434.1	1,809	0,723	0,724	1,615	1,584	1,579	1,578	1,530	1,626				
Plutella xylostella China KY435608.1	1,802	0,730	0,735	1,631	1,582	1,578	1,587	1,531	1,641	0,056			
Antheraea pernyi China NC023483.1	1,360	1,649	1,650	0,453	0,371	0,372	0,374	0,369	0,459	1,615	1,615		
Antheraea mylitta India MW115117.2	1,343	1,599	1,600	0,429	0,349	0,350	0,354	0,366	0,440	1,552	1,549	0,287	
Opsiphanes invirae Brazil NC027917.1	0,676	1,727	1,720	1,254	1,273	1,268	1,262	1,252	1,276	1,758	1,788	1,274	1,255

cause severe infections in beneficial insects, they generally remain asymptomatic in harmful insects (8-12). Therefore, more data are needed on iflaviruses found in agricultural and forest pest insects. In this study, the relationship between iflavirus isolates found in the NCBI database and whose complete genome analysis has been performed so far from pests of the order Lepidoptera was evaluated according to the Kimura-2 parameter and amino acid tree. The Kimura-2 parameter data obtained as a result of the study and the phylogenetic analysis results supported each other. Accordingly, iflaviruses isolated from lepidopteran species, especially forest pests, showed a great clustering with each other. However, some of the iflaviruses isolated from the agricultural pest lepidopteran species were more similar to each other and some of the isolates in the forest group. Although *Helicoverpa armigera* and *Spodoptera exigua* are important agricultural pests, are in the same family (Noctuidae), the iflaviruses isolated from them are quite far from each other. Helicoverpa armigera iflavirus isolate clustered close to the forest pest Lymantria dispar, Antheraea pernyi, Antheraea mylitta, Thaumetopoea pityocampa, and Heliconius erato iflavirus isolates. In addition, Spodoptera exigua iflavirus Spain1 isolate is distantly related to other Spodoptera exigua iflavirus Spain2 and Korea isolates (16, 18, 19) and showed similarity only to Opsiphanes invirae iflavirus isolate. (23). In this study, nucleotide distance analyzes of iflavirus isolates isolated from harmful insects in the order Lepidoptera for the first time were determined using the Kimura-2 parameter. The obtained data supported the addition of a new genus of the *Iflaviridae* family and the inclusion of

Spodoptera exigua iflavirus Spain1 isolate and Opsiphones invirae iflavirus Brazil isolate into a new genus by detecting detailed morphological data.

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions: GBE contributed to the study conception, design and laboratory work. Writing the article (GBE). All authors read and approved the final manuscript. GBE; Gözde Büşra Eroğlu

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The effects CaO nanoparticles applications on *Onobrycis sativa* seedlings growth under mannitol stress

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Cite:Yazıcılar B, Koç Ü. The effects CaO nanoparticles applications on Onobrycis sativa seedlings growth under mannitol stress. Eurasian Mol Biochem Sci 2022;1(2): 6-10.

Received: 04 October 2022, Accepted: 28 October 2022 *DOI. 10.54672/ejmbs.2022.8*

Abstract

In our study, two *Onobrycis sativa* population (*Uzuntekne*, and *Barış)* were used as the material for the response to CaO-NPs nanoparticulate. More DNSA and proline were collected in these two Onobrycis sativa genotypes than in control seedlings while two genotypes were exposed to mannitol. Proline content highest at 150 mM mannitol and 1.5 ppm CaO while the minimum and maximum content was observed at 50–150 mM mannitol dosages. The collected of DNSA was greatly correlated with higher mannitol concentrations. Proline activities demonstrated an increasing trend against the increasing concentration of mannitol. In conclusion, the growth characteristics and physiological responses of Onobrycis sativa increased, depending on genotype, mannitol and CaO dosage in the media and their interactions.

Keywords: Nanoscience, CaO, proline, DNSA

Introduction

Nanoscience has influenced every field of science and technology, of which long-term agricultural sustainability is a significant part of these fields (1). Nanoparticles have unique agronomic traits, i.e., protect in response to plant disease and use water effectively, alleviate environmental hazards and impacts of environmental factors. It provides novel information and decreases the treatments of chemicals and increases nutrient utilization efficiency, which ensures environmentally friendly sustainable production (2,3). They can supply eliminate nutrient deficiencies in plants, and increase the resistance of plants to stress factors in this way. Several studies have been shown on the exogenous treatment of nanoparticles for plant regeneration and development, but its effect on seedling growth and regeneration *in vitro* is limited compared to exogenous treatments. CaO-NPs can use the sustainable crop

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production and agricultural industry with the support of many novel techniques in reversing oxidative stress symptoms caused by environmental stresses (4). Moreover, the impact of CaO-NPs on physiological, biochemical, and antioxidative activities in many plant species has also been not tested novelty. Tissue culture techniques are particularly beneficial in all areas of food science because these techniques can contribute to the agronomic improvement of plants by eliminating the difficulty in exogenous applications under uncontrolled conditions (5). The *Onobrycis sativa* is one of the most economic-growth forage legumes widely grown worldwide. It has been an important grain forage crop for livestock, environmental, nutraceutical attributes, and nutritional. *O. sativa* is cultivated for its honey production and is a valuable resource for pollinators (6,7). Farmers also benefit from its drought resistance in the field of drought and light-free draining soil, mainly due to its deep taproot. This study aimed to determine the seedlings regeneration, proline, and DNSA (3,5-Dinitrosalicylic acid) for *O. sativa* by testing different CaO concentrations *in vitro* medium.

Materials and Methods

Plant material

Plant material and CaO treatments: In our study, two *O. sativa* genotypes (*Uzuntekne*, and *Barış*) were used as the material for the response to CaO NPs nanoparticulate and mannitol. Seeds were surface sterilized with 22% NaOCl for 30 minutes and disinfected three times with sterile distilled water. Then, seeds were grown in plates including full MS medium (8) from two different CaO NPs concentrations containing 0.5, and 1.5 ppm CaO NPs nanoparticulate and 50 and 150mM mannitol.

DNSA: For DNSA determination, 10 mg of tissue per seedling was ground in a mortar, homogenized in 1 mL of 80% ethanol, and centrifuged at 5000 × g at 4 °C for 10 min. Supernatants were transferred into other tubes and the pellets were homogenized again in 0.5mL of 80% ethanol and centrifuged as above. The second supernatant was added to the first. Total DNSA were measured by a modified method by Watanabe et al. (9). One milliliter of the extract was reacted with 3 mL of freshly prepared anthrone reagent (50 mg anthrone + 50 mL of 95 % H_2SO_4) at 100 °C for 10 min. After cooling on ice, the total DNSA was determined at 620 nm with a spectrophotometer using glucose as a standard.

Proline estimation: The proline amount was determined with the producer of Bates et al. (10). Seedling samples (100 mg) were powdered in 5 mL of 3% aqueous sulfosalicylic acid and centrifuged at 4 °C for 15 min at 4800 × g. Extract (2 mL) was mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in test tubes. Samples were boiled for 1 h at 100 °C. The reaction was terminated in an ice bath and 4 mL of toluene was used for the reaction of the mixture extraction. The absorbance of the color reaction product was measured at 520 nm using toluene as a blank. The proline concentration was determined from a calibration curve.

Statistical Analysis: Each experiment was replicated three times. Analysis of variance was carried out using a two-way ANOVA test using SPSS 13.0 and means were compared by the Duncan test at the $P<0.05$ confidence degree.

Results

Proline content: CaO treatments caused different effects on the proline content. There was a detectable difference among genotypes and concentrations. Proline amount displayed high variation between tested samples for CaO and mannitol applications, ranging from 0,130 to 1.195 mg/g FW. The proline amount in 150 mM Mannitol/1.5 ppm CaO of the *Barış* genotype was higher than that of the other

concentrations under CaO and mannitol treatments, which peaked at 1.195 mg/g FW. The highest content was found (1.153 mg/g FW) from seedlings treated with 150 mM Mannitol/0.5 ppm CaO in the *Uzuntekne* genotype (Figure 1). There was also a detectable difference in proline content between mannitol and CaO treatments.

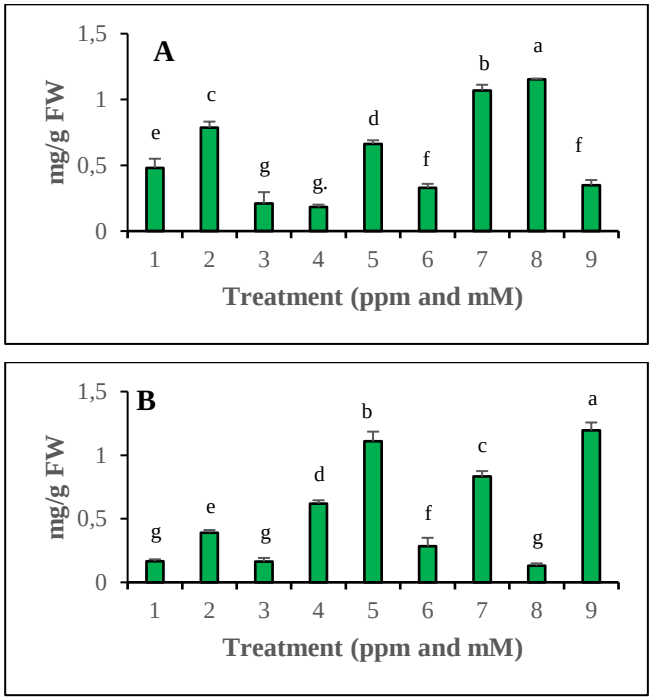

Figure 1. Changes in proline contents of *O. sativa* genotypes treated with the mannitol stress and CaO-NPs. A: *Uzuntekne*, B: *Barış*. Lower-case letters for the study indicate statistically significant differences between the groups at *P<0.05*. Bars mean SE. (1: Control, 2:0.5 ppm CaO, 3: 1.5 ppm CaO, 4:50 mM mannitol, 5: 150 mM mannitol, 6: 50 mM mannitol/0.5 ppm CaO, 7: 50 mM mannitol/1.5 ppm CaO, 8: 150 mM mannitol/0.5 ppm CaO, 9: 150 mM mannitol/1.5 ppm CaO).

DNSA: Figure 2 displays that DNSA were highly affected in the seedlings stage of two *O. sativa* genotypes in presence of 0.5 ppm, 1.5 ppm CaO-NPs and 50 mM mannitol, 150 mM mannitol treatments. DNSA revealed an extent range of variation between tested samples for CaO-NPs ve mannitol applications, ranging from 0.024 to 1.498 mg/g FW of the *Uzuntekne* genotype. The highest content was obtained from seedlings treated with control in the *Barış* genotype. The *Uzuntekne* genotype in 50 mM mannitol/1.5 ppm (1.498 mg/g FW) indicated the best result in standard CaO-NPs and mannitol for DNSA compared to the other concentrations. Although the maximum DNSA content was found in the treatments of control and 50 mM mannitol/1.5 ppm CaO in seedlings, the lowest DNSA content was found in seedlings for 0.5 ppm CaO-NPs (Figure 2).

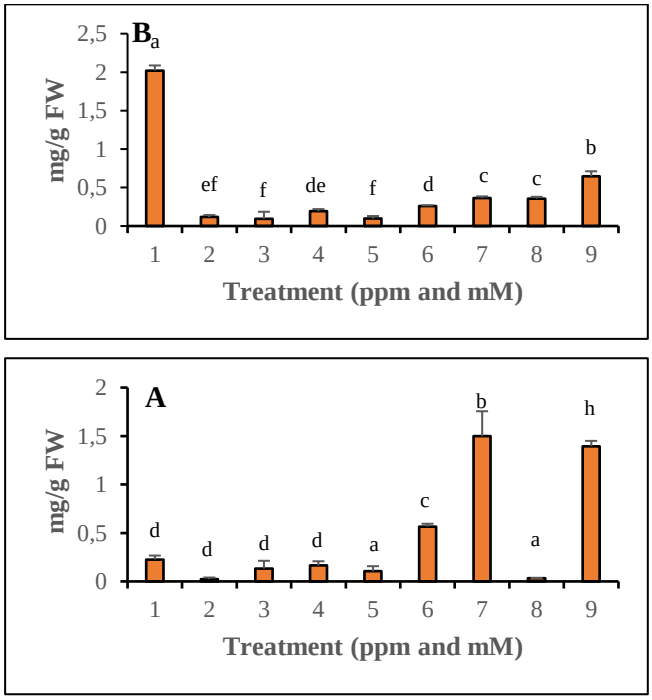

Figure 2. Changes in soluble sugar content of O. sativa genotypes treated with the mannitol stress and CaO-NPs. A: *Uzuntekne*, B: *Barış*. Lower-case letters for the study indicate statistically significant differences between the groups at P<0.05. Bars mean SE. (1: Control, 2:0.5 ppm CaO, 3: 1.5 ppm CaO, 4:50 mM mannitol, 5: 150 mM mannitol, 6: 50 mM mannitol/0.5 ppm CaO, 7: 50 mM mannitol/1.5 ppm CaO, 8: 150 mM mannitol/0.5 ppm CaO, 9: 150 mM mannitol/1.5 ppm CaO).

Discussion

Ca are a major essential nutrient for growth and development in plants. It induces enzymes, plant vegetative biomass, and photosynthesis ratio, and increases biochemical reaction. CaO-NPs (Ca^{+2}) is important elements and several biochemical and molecular changes during the plant cycle (11,12). In this study, applications of CaO highly affected the seedlings regeneration, development, proline, and DNSA. CaO at

two doses was tested in vitro on seedlings tissues in the MS media in the combination with 4 mg L-1 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.125 mg kinetin including 0.5, 1.5 ppm CaO NPs nanoparticulate. The observed improvement in plant growth traits in vitro culture due to the supplementation of CaO-NPs is matched with previous studies (4,13). In seedlings samples, proline was greatly increased under drought stress applications and the impacts of CaO-NPs on proline content are well linked to the mannitol-resistance ability. High-concentration CaO and mannitol-treated *O. sativa* seedlings showed a significant decrease in stress (Figure 1). Proline content has been exhibited to have defensive impacts in response to mannitol stress (14). These results also indicated a raised collection of proline amount in mannitol-tolerant seedlings as compared to control seedling samples of two samples. Similarly, Nayyar et al. (15) found that varied mannitol treatments increased proline levels in wheat and maize. Soheilikhah et al. (16) obtained similar results for *Carthamus tinctorius L.* varieties callus cultures under salt and mannitol stress. In terms of DNSA, CaO-NPs at the highest concentration increased the content of these molecules (Figure 2). Both proline and DNSA content maintain a certain point of metabolic equilibrium in the plant cells, and when the plant is subject to external factors, this equilibrium will be unstable. DNSA at low concentrations of CaO, increased significantly in an exposure time-dependent manner. On the other hand; High-concentration CaO treatment can significantly increase the content of DNSA, which could be due to the injury of metabolic equilibrium, thus blocking enzyme activity. Similar results were observed in DNSA amounts in the present study. Results are matched with those published by Signh and Kumar (17) in the study on mannitol for *Eucalyptus tereticornis*. Their results demonstrate that as the concentration of nanoparticles, DNSA amount decreased. In conclusion, the growth characteristics and physiological responses of *O. sativa* increased, depending on genotype, mannitol, and CaO dosage in the media and their interactions. Proline and DNSA in *Barış* seedlings grown under osmotic stress were exhibited to be greater than those in *Uzuntekne* seedlings. These parameters may be employed as criteria for assessing drought resistance.

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions: BY and ÜGK contributed to the study conception, design and laboratory work. Writing the article (BY and ÜGK). All authors read and approved the final manuscript. BY; Büşra Yazıcılar, ÜGK; Ümmü Gülsüm Koç.

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Eurasian Journal of Molecular and Biochemical Sciences

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Melatonin and cisplatin synergistically enhance apoptosis via autophagy-dependent alteration of P53 transcription in human colorectal cancer cells

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Cite:*Polat S, Topal H, Celep N.A., Erbaş E, Kara A. Melatonin and cisplatin synergistically enhance apoptosis via autophagy-dependent alteration of P53 transcription in human colorectal cancer cells. Eurasian Mol Biochem Sci 2022;1(2): 11-18.*

Received: 10 August 2022, Accepted: 28 October 2022 *DOI. 10.54672/ejmbs.2022.9*

Abstract

Cisplatin is one of the most general chemotherapeutic agents used to treat various cancers, including colorectal and breast cancer. because cisplatin has some adverse effects including cardiotoxicity and hepatoxicity, it is usage limited. Melatonin is a natural product that responsible for regulator of circadian rhythms and has anti-cancer potential. However, its synergistic effects with melatonin and cisplatin, its efficacy in cancer cell death, its mechanisms and biological targets are not well understood. In this study, it was aimed to determine the synergistic activity of cisplatin with melatonin in colon cancer cell death through apoptotic and autophagic mechanisms. In the present study, we found that melatonin with cisplatin treatment did not affect the cytotoxicity, but cisplatin increased in 24 h incubation period. Melatonin and combined treatment of melatonin and cisplatin also increased the cytotoxicity in 48h incubation period. It was observed that cisplatin treatments used together with melatonin and melatonin inhibited the mitogen activity of colon cancer cells. In addition, combined treatment of cisplatin and melatonin and single treatment of cisplatin increased both apoptosis and autophagic cell death. The results revealed that the use of melatonin with combined cisplatin has been shown to increase the apoptosis and autophagic cell deaths via P53 gene activation.

Keywords: Melatonin, cisplatin, colorectal cancer, apoptosis, autophagy

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Introduction

Colorectal cancer is the third most common type of cancer worldwide and is the second leading cause of

cancer-related deaths. In addition, while the incidence of colon cancer is increasing with obesity worldwide (1) the effectiveness or synergistic treatment of natural products molecules in the treatment of cancer has been emphasized in recent years (2) Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesized and secreted by the pineal gland in the body, and is an indolic compound that plays a central role in the regulation of circadian and seasonal biorhythms in humans (2). Melatonin has shown chemotherapeutic potential in many cancer types. It can also rise the efficacy of anticancer drugs (eg, cisplatin, epirubicin) by regulating many different signaling pathways (3). Thus, melatonin has been recognized as a potential complementary product in chemotherapy as well as to reduce the negative effects of anticancer drugs (2). Nevertheless, the mechanisms of melatonin synergize with anticancer drugs are still unclear. An occurring any DNA damage in the cell, many pathways make play role in the cell (4). These damages activate pathways that function at cell checkpoints and slow down the transition to S phase by inhibiting the progression of cells towards G1 and G2 phases. DNA Damages cause an excessive expression of the p53 tumor suppressor gene that regulates the cell cycle. TP53 is a very short-lived protein (5). The p53 gene, which ensures the stability of the genome, is activated in DNA damage, preventing the cycle from G1 to S, thus providing the cell with the time necessary for the repair of the damage. With the increase in p53 expression, transcription of many genes is also stimulated. If the damage is repaired, the Murine double minute 2 (mdm2) gene, which is an important regulator of the p53 tumor suppressor gene, is activated. With its E3 ubiquitin ligase activity, the MDM2 protein recognizes p53 from its N-terminal trans-activation domain, causing its proteosomal degradation and inhibiting the transcriptional activity of p53 (6). In this way, it regulates the cell cycle negatively. When DNA damage occurs in the cell, structural changes such as acetylation and phosphorylation occur at the binding site of MDM2 protein to p53, and MDM2 cannot bind p53. Therefore, free p53 cannot stop the cycle. If the damage cannot be repaired, p53 induced apoptosis cell death the in the cell (7). Additionally, cell cycle progression is controlled by cyclin-dependent kinases (Cdk), which are the catalytic partners of cyclins that maintain the cycle. Cdk activity that is not regularly controlled causes increased cell proliferation and genomic instability. Three different Cip/Kip (Cdk Inhibitory Protein / Kinase Inhibitory Protein) family CDKIs have been identified that regulate Cdk activity and stimulate cell cycle suppression. These genes are p21Cip1 (CDKN1A), p27Kip1 (CDKN1B) ve p57Kip2 (CDKN1C) (8). The first cloned Cip/Kip member, CDKI, is p21 and is responsible for G1 suppression in the response to DNA damage. p27 is an important molecule that regulates growth in response to antimitogenic signals. p57; It has 40% homology with p27 and is involved in proliferation and differentiation (8). According to the results of research conducted in recent years; It is known that p21 induces "growth arrest", p57 ensures the continuation of "growth arrest" and p27 stimulates differentiation genes. Mutational inactivation of Cip/Kip family CDKIs is very rare (9,10).

In the present study, we examined the potential of melatonin to enhance the efficacy of cisplatin in Colorectal cancer cell lines. We demonstrate that the combination of melatonin and cisplatin remarkably induces autophagfy and apoptosis in the Colorectal cancer cells by altering P53 mRNA. We also investigated the synergic effects of melatonin and cisplatin in the colorectal cancer cells.

Materials and Methods

Cells and culture conditions: Human Colorectal cancer cells (HT-29) were purchased from the Tarım ve Orman Bakanlığı Şap Enstitüsü (Ankara, Turkey). The cells were maintained as mono-layer cultures in 75-cm2 plastic culture flasks in Dulbecco's modified

Eagle's medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA), 2%L glutamine (Gibco, USA), %1 penicillin (20 units/mL) and streptomycin (20 lg/mL) (Sigma-Aldrich, USA) at 37°C in a humid atmosphere containing 5% CO_2.

Drug treatment: Human Colorectal cancer cells (HT-29) were seeded in a 96-well plate at a density of 1500 cells/well in 100-µL of complete culture medium and left attach overnight. After 24 h and 48h, melatonin (dosing at 5-µM and 10-µM) (Sigma Aldrich, USA) and/or cisplatin 50-µM was added (KoçakFarma, Turkey). Melatonin was dissolved at 1M stock solution in ethanol (Sigma-Aldrich, USA) and the corresponding alcohol concentration (ethanol at a final concentration lower than 0.0001%) was added to control cells. Cisplatin was diluted with DMEM solution.

Measurement of cellular proliferation: After 24h and 48h of incubation, MTT reagent was added at a final concentration of 0.5 mg/mL and allowed to react for 3h. Then, MTT solution (100-µl) was added and incubated in the dark at 37 °C for 20 minutes. The absorbance was measured at a spectrophotometer microplate reader µ-Quant, BioTek Instruments (Winooski, Vermont, USA) at a wavelength of 570 nm using absorbance at 690 nm as reference wavelength.

Tunel assay: After the 24h and 48 h incubation of cell on poly-L-lysine coated slides in the 10 cm petri dishes, the slides were fixed with methanol solution at -20 °C for 10 min and then washed with PBS. The apoptotic cells were detected by terminal Transferase dUTP Nick End Labeling (TUNEL Promega G7130) method. For fixation, ethanol solution at -20° was used and then slides dropped in 3% hydrogen peroxide. Then, the cells were treated with 0.1% Triton X-100 for 10 min for permeabilization. After 3 times washing with PBS, 1% bovine serum albumin (BSA) in PBS solution was used for blocking of non-specific bindings. The cells were incubated with terminal deoxynucleotidyl transferase enzyme (Tdtenzyme) for 1 h at 37°C. Then cells were applied with Converted Pod solution for 30 min. The cells were stained with Harris hematoxylin for counterstaining. Cells were mounted with aqueous media. The apoptotic cells were counted using light microscope (Nikon eclipse i50, Japan).

Tunel positive cell evaluation: For the apoptotic (tunel positive cells), Stereological Optic Fractionator Frame method was used to compare the tunnel results between groups. These analyzes were performed under stereology workstation system (BioPrecision MAC 5000controller system) and stereology software (Stereo Investigator version 9.0, Microbrightfield, Colchester, VT) attached light microscope (Leica DM4000B, Tokyo, Japan). In our study, tunnel positivity on HT-29 cell preparations was calculated using the "Unbiased Counting Frame and Fractionator" method, and the positive cell density in each preparation belonging to all groups was calculated according to the following formula (Fig. 1): PHY = PHS/(CA x RS), PHY; Positive cell density per µm² area, PHS; positive cell count, CA; frame area (µm²) and RS; number of frames. The data obtained are based on duplicate measurements for each group, and 4 parallel preparations from each group were stained.

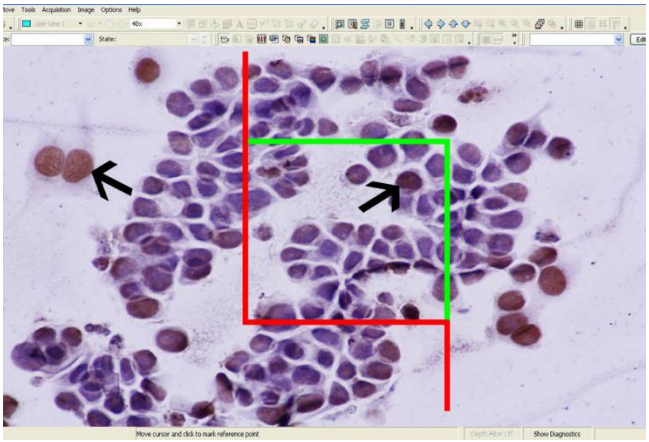

Fig 1. Evaluation of tunnel positivity with stereological "Optical Fractionator Frame" method.

Quantitative Real Time PCR (qRT-PCR) Analyzes

RNA isolation: The total cellular RNA was isolated from HT-29 cells and purified with the Nucleospin RNA Kit (Qiagen RNA Mini Kit, USA) following the manufacturer's instructions. The concentration and purity of the isolated total RNA were determined by spectrophotometric method. For this purpose, Nanodrop device designed for DNA/RNA measurements, capable of measuring in micro volume was used (µ-Biotek, USA) and RNA concentration and purity were calculated automatically with the software on the device according to 260/280 nm values, and the results were determined as ng/µl.

cDNA Synthesis: For cDNA Synthesis, 140 ng RNA was denatured at 65°C for 10 min and reverse transcribed for 50 min at 45°C with cDNA Synthesis kit (Qiagen, USA) in a final volume of 20-µL. After determination of obtained cDNAs concentrations by spectrophotometric method (µ-Biotek Nanodrop, USA), relative quantitation analyzes of cDNAs were started with qRT-PCR (Roche, German).

qRT-PCR analysis: For the mRNA expression levels of BECLIN1, ATG4, TP53, MDM2, CDKN1A, CDKN1B and LC3 genes were evaluated the using quantitative real time PCR (Roche Light Cycler 480 Real-Time, German). qRT-PCR was performed in 20 µl total volume (5µl cDNA, 8 µl ddH2O, 5 µl Probe Master mix 2 µl primers). In qRT-PCR, 4 standards were used to obtain the Costom Assay PCR program's principle Absolute Quantification value. Then, Absolute Quantitative analysis was performed and the values given by the device to the samples as BECLIN1, ATG4, TP53, MDM2, CDKN1A, CDKN1B and LC3 were obtained based on the standards.

Statistical analysis: Normal distributed values form MTT, tunel and qRT-PCR analyses were evaluated the Duncan Post hoc test after one-way analysis of variance (ANOVA) analysis. The significance value (P) was accepted as 0.05. in qRT-PCR analysis, Glucose 6-phosphate dehydrogenase (G6PD) was used as a housekeeping gene for the determination of BECLIN1, ATG-4, TP53, MDM2, CDKN1A, CDKN1B and LC3 gene expression levels and were normalized by taking the target Gene/reference gene ratio.

Results

Cytotoxicity evaluation: In the MTT analysis, it was determined that the cytotoxic effect of cisplatin treatment was significantly higher in the groups incubated for 24 hours ($p<0.05$). In the 48-h incubation groups, cisplatin (50µM) + melatonin (5µM) and Cisplatin (50µM) treatments were showed a similar effect on the cells. Therefore, the cytotoxic effect of melatonin doses was found to be similar to the control group (Table 1 and Fig. 2).

Table 1. Effect of Mel and/or Cis on Cytotoxicity in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM) for Tunel staining.

Groups	24h	48h
Control	0.854±0.08[a]	0.751±0.11[a]
Mel-5µM	0.839±0.13[a]	0.696±0.23[b]
Mel-10µM	0.863±0.21[a]	0.728±0.19[a]
Cisplatin	0.728±0.14[b]	0.622±0.12[b]
Mel-5µM-Cis	0.847±0.17[a]	0.649±0.15[b]

[(a,b)] the Letters indicate statistical difference between columns.

Tunel Analysis Results: In the evaluation of apoptotic cells, there was no significant difference between tunel positive cell densities of Control, Mel-5µM and HT-29 cells incubated for 24 hours with Mel-10µM- Cisplatin (50-µM) treatments ($P>0.05$), while significant tunneling was observed in HT-29 cells only cisplatin applied. It was determined that there was a significant increase in positive cell density ($P<0.05$).

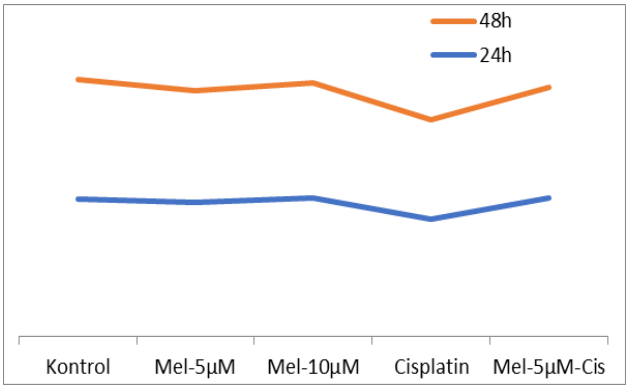

Fig. 2. Synergistic cytotoxicity of melatonin (Mel (5µM) and Mel (10µM)) and Cisplatin (Cis(50µM)) co-treatment (Mel (5µM) +Cis (50µM)) in HT-29 cell lines incubated for 24h and 48h.

In addition, while the tunel positive cell density of Cis-50 µM and Mel-5µM+Cis-50 µM groups was significantly higher than the other groups in the 48-hour incubation period ($P<0.05$), there was no found any statistical differences between Mel(5µM), Mel (10µM), and control groups ($P>0.05$). Tunel positive cell densities for all groups were presents in Table 1 and seen in Table 2 and Fig 3.

Table 2. Effect of Mel and/or Cis on Tunel-positive cells in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM) for Tunel staining.

Groups	24h	48h
Control	0.101±0.04[a]	0.151±0.02[a]
Mel (5µM)	0.115±0.02[a]	0.167±0.03[a]
Mel (10µM)	0.146±0.03[a]	0.203±0.04[a]
Cis (50µM)	0.235±0.04[b]	0.426±0.07[b]
Mel (5µM) +Cis (50µM)	0.165±0.07[a]	0.278±0.06[c]

[(a,b,c)] the Letters indicate statistical difference between columns.

mRNA expression levels: In the TP53 mRNA expression analysis, the expression levels of Mel (5µM) and Cis (50µM) groups in HT-29 colon cancer cells incubated for 24 hours were higher than the other groups, while the expression levels of Mel (10µM) and Mel (5µM)+Cis(50µM) groups were found to be nearly similar to the Control group. In 48h incubation period, TP53 mRNA expression levels of Mel (5µM) and Mel (5µM)+Cis(50µM) groups were increased compared to control group. Other groups were found nearly similar to control group (Table 3 and Fig 4).

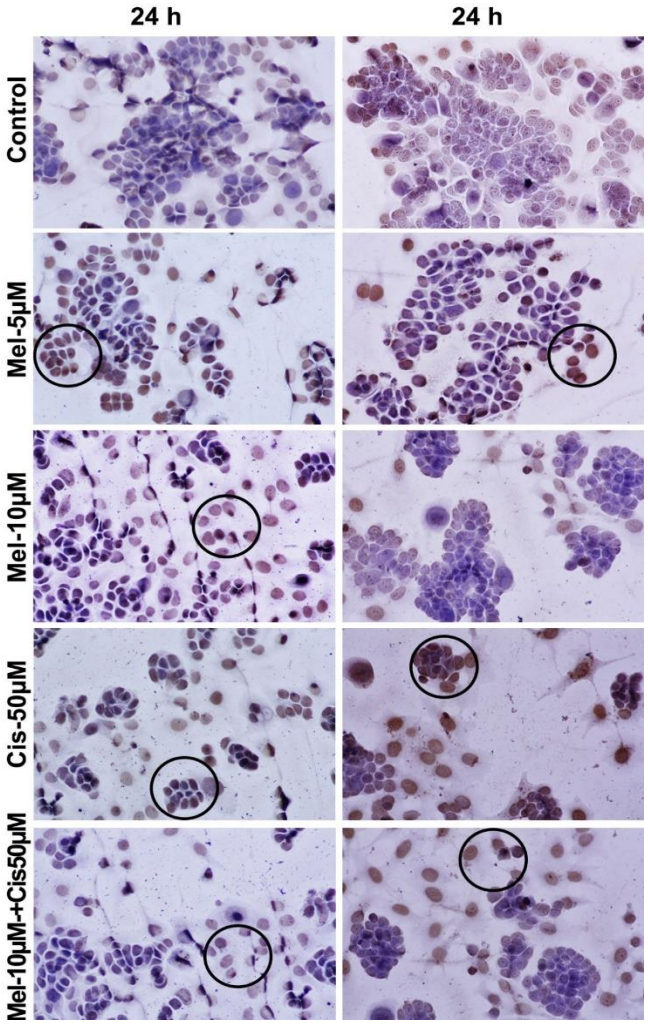

Fig 3. Illustrations of Mel and/or Cis on Tunel-positive cells in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM) for Tunel staining.

In the MDM2 mRNA expression, the expression of Mel(5µM) and Cis(50µM) groups were higher in HT-29 colon cancer cells incubated for 24 hours, also the expression level was found higher in the Mel-10µM group than the control group, but lower in the Mel (5µM)+Cis(50µM) group than the control group. In the 48h incubation groups, the highest mRNA expression was observed in the Mel (5µM)+Cis(50µM) group, while the mRNA expression levels in the other groups decreased (Table 3 and Fig 4).

Table 3. Effect of Melatonin and/or Cisplatin on gene expression levels in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM).

	Groups	TP53/ G6PD	MDM2/ G6PD	CDKN1A/ G6PD	CDKN1B/ G6PD	BECLIN1/ G6PD	ATG-4/ G6PD	LC3/ G6PD
24 h	Control	0.184	1.636	0.154	0.189	0.349	0.095	0.013
	Mel (5µM)	0.472	3.317	0.748	0.399	0.678	0.202	0.032
	Mel (10µM)	0.241	2.694	0.398	0.188	0.548	0.124	0.065
	Cis (50µM)	0.412	4.313	2.720	0.164	0.537	0.139	0.061
	Mel (5µM) + Cis (50µM)	0.159	1.184	0.220	0.108	0.220	0.051	0.011
48 h	Control	0.175	1.924	0.350	0.365	0.473	0.140	0.037
	Mel (5µM)	0.160	0.777	0.096	0.295	0.295	0.074	0.019
	Mel (10µM)	0.232	1.427	0.210	0.308	0.368	0.107	0.021
	Cis (50µM)	0.402	1.699	1.139	0.337	0.570	0.203	0.163
	Mel (5µM) + Cis (50µM)	0.300	2.797	1.234	0.222	0.483	0.193	0.140

The values of relative mRNA expression levels were normalized with internal control (G6PD)

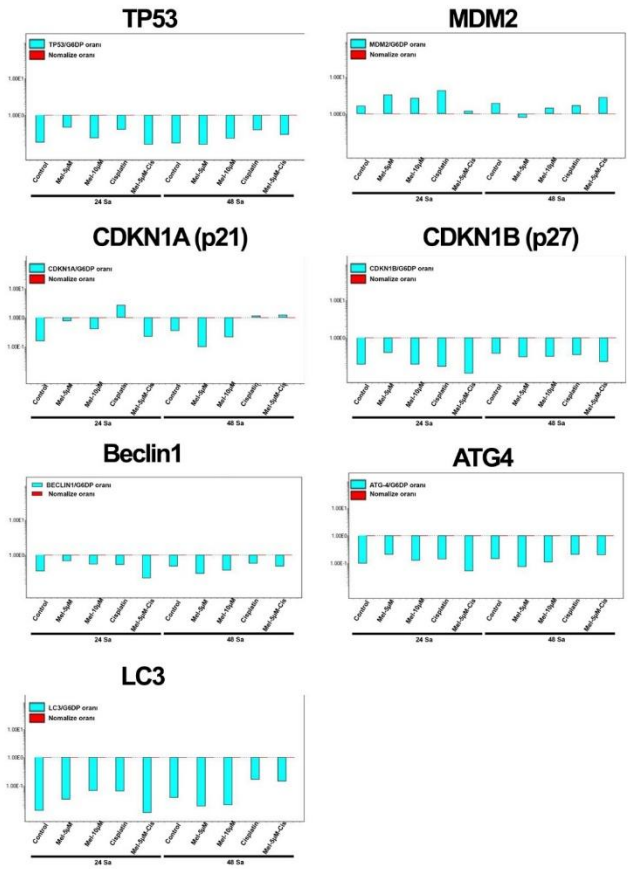

Fig 4. Effect of Melatonin and/or Cisplatin on relative gene expression levels in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM).

In the analysis of CDKN1A mRNA expression, the highest expression level was found in the Cis (50µM) group in the HT-29 cells incubated for 24h. Although, it was observed an increase in the Mel (5µM) group, the increase in the other groups was close to the control group incubated for 24h. Also, the highest values were found in Cis (50µM) and Mel (5µM) +Cis (50µM) groups in 48 hours incubation groups. It was observed that the increase in the other groups was close to the control group value (Table 3 and Fig 4).

In CDKN1B mRNA analysis, an increase was observed in the expression level in the Mel (5µM) group with 24-h incubation, while the expression levels of the other groups were found to be close to the Control group. Also, mRNA levels of CDKN1B of all groups incubated for 48 h were decreased compared to the Control group (Table 3 and Fig 4).

In the mRNA expression analysis of BECLIN1 gene, had the highest expression value was found in Mel-5µM, Mel-10µM and Cis groups in HT-29 cells incubated for 24 hours. Also, mRNA expression level of BECLIN1 was decreased in Control and Mel (5µM) + Cis (50µM) groups. On the other hand, mRNA

expression level of BECLIN1 was increased in Control, Cis (50μM) and Mel (5μM) + Cis (50μM) groups in HT-29 colon cancer cells incubated for 48h, while the expression value of Mel (5μM) and Mel (10μM) groups was found to be lower than in the Control (Table 3 and Fig 4).

In the analysis of ATG-4 mRNA expression, Cis (50μM) and Mel (10μM) groups were higher than the Control and other groups in colon cancer cells incubated for 24 hours. The lowest value for incubated 24 h groups was found Mel (5μM) + Cis (50μM) group. In the ATG-4 mRNA expression levels of HT-29 colon cancer cells incubated for 48 hours, the highest expression was observed in Cis (50μM) and Mel (5μM) + Cis (50μM) groups, while the lowest value was found in the Mel (5μM) group (Table 3 and Fig 4).

In the LC3 mRNA expression analysis, the expression levels were found higher in the Mel (10μM) and Cis (50μM) groups in the 24-hour incubation groups. The mRNA expression levels of other groups were close to the control group. Also, In the group incubated for 48 hours, an increase was determined in the Cis (50μM) and Mel (5μM) +Cis (50μM) groups. In the Mel (5μM) and Mel (510μM) groups, a decrease in their expression was determined compared to the Control group (Table 3 and Fig 4).

Discussion

The goal of chemotherapy is to induce cancer cell death without damaging non-cancerous cells or tissues. Cisplatin is an essential chemotherapeutic reagent for the treatment of many tumors including colon cancer as well as many other types of cancer, but its many side effects limit its indication. Therefore, researchers are seeking to improve the therapeutic efficacy of cisplatin therapy for minimizing side effects of cisplatin. Melatonin has also been reported to show protective effects against various anti-cancer drugs (11). Thus, melatonin seems to be one of the most effective complementary components that can meet this requirement and increasing the sensitivity of cancer cells to cisplatin while providing protection against toxicity caused by cisplatin (12).

Colorectal cancer is one of the most common types of cancer (13). The study was investigated the effects of melatonin and/or cisplatin on cell cytotoxicity, apoptotic cell death and autophagy on colorectal cancer. In the study, HT-29 cell line was used and different concentrations (5, 10 nM) of melatonin and cisplatin (50 μM) were treated for 24 and 48 hours. In the result, administration of melatonin and/or cisplatin significantly decreased cell viability. Previous studies supports the therapeutic properties of melatonin on cancer cells (14). Also, some studies suggested that melatonin affects mitochondrial function by reducing ATP synthesis, triggering OS to encourage death in cancer cells, and blocking telomerase activity (15).

In the results of the tunnel analysis, it was observed that the melatonin and cisplatin treatments were therapeutically effective on HT-29 cells and increased the number of apoptotic cells. Combined administration of melatonin and cisplatin has been shown to be effective in osteosarcoma cells (16). In our study, the administration of melatonin and/or cisplatin was also found to be more effective when combined to use.

Expression levels of BECLIN1, ATG4, TP53, MDM2, CDKN1A, CDKN1B and LC3 genes, which are related to apoptotic and autophagy, were analyzed by quantitative PCR method. The BECLIN1 gene is one of the important genes involved in autophagy (17). In our study, mRNA expression level of Beclin1 was found higher in the 24 and 48h incubated HT-29 colorectal cancer cells treated with melatonin and/or cisplatin. Although ATG4 and LC3 genes are also genes involved in autophagy, autophagic cell death in the cancer (18). In the present study, the expression levels of ATG4 and LC3 were found increased in the 24 and 48 h melatonin /and or cisplatin incubated HT-29 cancer cells.

In the study, an increase was observed in the mRNA level of the p21 (CDKN1A) gene in the melatonin and cisplatin incubated groups compared untreated groups. The p21 gene is synthesized by the p53 gene and provides the synthesis of CDKs that control the cell cycle (19). Inhibitors that control cell division are called cyclins and one of cyclins is p27 (CDKN1B) gene, which is also a suppressor of TGFB, which controls the G2/M transition, which controls the cell cycle. In our study, it was observed that melatonin and cisplatin treatment increased the mRNA levels of p27.

The p53 gene is an important protein that is responsible for preventing tumor growth (20). In our study, an increase was observed in the treatment of melatonin and cisplatin in the level of the p53 gene. This shows that the treatment of melatonin and cisplatin trigger the colorectal cancer cell to apoptosis. Melatonin and cisplatin applications were also found to decrease at the level of MDM2, which is known as an inhibitor of TP53 proteins.

As a result, it was observed that the separate and combined treatment of melatonin and cisplatin provided a cytotoxic effect in HT-29 cells with colorectal cancer, it stimulated apoptotic cell death and increased autophagic gene expressions. therefore, it has been shown that melatonin and cisplatin applications can have therapeutic properties for colorectal cancer.

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions: SP, HT, EE NAC, AK contributed to the study conception, design and laboratory work. Writing the article (SP, HT, AK). All authors read and approved the final manuscript. SP; Süleyman Polat, HT; Halime Topal, NAC: Nevra Aydemir Celep EE: Elif Erbaş, AK; Adem Kara.

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Eurasian Journal of Molecular and Biochemical Sciences

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Determination of the potential of 5-Hydroxy-L-tryptophan and L-tryptophan as therapeutic agents for prostate cancer

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Cite:*Tozlu Ö.Ö, Yüksel N, Gezmiş T, Tanas A. Determination of the potential of 5-Hydroxy-L-tryptophan and L-tryptophan as therapeutic agents for prostate cancer. Eurasian Mol Biochem Sci 2022;1(2): 19-25.*

Received: 9 September 2022, Accepted: 27 October 2022 *DOI. 10.54672/ejmbs.2022.10*

Abstract

Prostate cancer is the second leading cause of death from cancer in men. Androgen deprivation therapy (ADT) is used as the standard treatment in prostate cancer, and this treatment has undesirable side effects over time. There is a need for more effective, safe compounds that occur naturally under the influence of these undesirable limitations. In this study, the anti-cancer potentials of 5-Hydroxy-L-tryptophan (5-HTP) and L-tryptophan, which are thought to have various inhibitory potential on cancer and its mechanisms, were studied. The study results showed that 5-HTP has a significant inhibition in prostate cancer cells. This study is an important preliminary screening for new and effective molecule trials. In order to develop a possible treatment strategy and to use these molecules as new therapeutic agents, further studies are needed in order to obtain more comprehensive data on the mechanisms of action of these molecules and to investigate their possible effects in other cancer types, and the obtained data should be supported by these studies.

Keywords: Prostate cancer, 5-Hydroxy-L-tryptophan, L-tryptophan , MTT assay, LDH release

Introduction

Prostate cancer, which is the most diagnosed cancer among men in Europe and America, is the second most common cause of death from cancer in men. It is observed that mortality rates have decreased due to the widespread use of prostate-specific antigen (PSA) secreted in the prostate, which is thought to contribute to the motility of spermatozoa, and developments in transrectal ultrasound-guided prostate needle biopsy (1–3).

It has long been known that prostate cancer is dependent on androgen for its growth and progression. Androgens, produced in the testicles, adrenal glands, and prostate gland, are essential for normal development and function of the prostate and prostate

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cancer proliferation. Therefore, androgen deprivation is an effective therapeutic strategy widely used in clinical practice and has become the standard treatment for this disease. Androgen deprivation therapy (ADT) is used to suppress androgenic effects and therefore prevent progression of prostate cancer (4,5). However, most patients develop resistance to metastatic castration after several years of ADT therapy and progress to prostate cancer (6). In addition, since androgens affect many other organs besides the prostate, according to the mechanism of action of the drug used, ADT may cause decreased libido, erectile dysfunction, hot flashes, loss of bone density, bone fractures, loss of muscle mass and physical strength, changes in blood lipids, insulin resistance, weight gain, It can have various side effects such as burnout and gynecomastia. Today, many new drugs are introduced to the market for use in the treatment of prostate cancer. Due to these limitations and adverse effects of current standard treatments, the search for safer and more effective molecules based on naturally occurring compounds is emerging.

L-Tryptophan is an essential aromatic α- amino acid and is required in the diet of children and adult humans. It serves as a precursor for important biomolecules such kynurenic acid, nicotinamide adenine dinucleotide, serotonin, melatonin, tryptamine, and niacin in addition to being a necessary amino acid for protein synthesis (7–9).

5-Hydroxy-L-tryptophan (5-HTP), a serotonin pathway metabolite of L-tryptophan in the brain that regulates serotonin levels. As a result, 5-HTP is a key player in the serotonin pathway. Additionally, 5-HTP is a naturally occurring aromatic amino acid that has a variety of antioxidant properties (10–13). Additionally, 5-Hydroxy-l-tryptophan (5-HTP) is a well-known dietary supplement that has taken the place of l-tryptophan (l-Trp) as a treatment for depression (14,15), fibromyalgia's incapacitating symptoms (16,17), weight loss assistance (18), headache prevention (19), and assistance for insomniacs (20,21). Taking together all these data, the aim of this study was to elucidate the anticancer potentials L-tryptophan and 5-HTP on prostate cancer cells. Therefore, L-tryptophan and 5-HTP were screened for their cytotoxicity using 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay and LDH release assay. Also, they were examined for antioxidative and oxidative status using total antioxidant capacity (TAC) and total oxidant status (TOS) assay.

Materials and Methods

Cell culture: PC3 and DU-145 cells cell line were kindly provided from Dr. Ömer Faruk Karataş (Erzurum Technical University). The cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco, Life Science, USA) (1:1) medium supplemented with 10% fetal bovine serum (PAN Biotech®), 1% streptomycin/penicillin and maintained at 37°C in a 5% CO2 incubator.

MTT Assay: For MTT assay, $1x10^4$-$1x10^5$ cells were seeded in 96-well plates and kept under appropriate culture conditions (37 °C, 5% CO2) for 24 h for cell attachment. Then, cells were incubated with different concentrations of 5-HTP and L-tryptophan for 48h. After incubation period, MTT solution (5 mg/ml MTT in PBS; Sigma-Aldrich®, Germany) was added and incubated for 3 h. Then, dimethyl sulfoxide (DMSO) (Merck®, Germany) is used to dissolve formazan crystals. In a microplate reader, the optical density was measured at 570 nm of wavelength (Synergy-HT; BioTek Winooski, VT, USA). Cells were given a treatment with 0.1% (w/v) Triton X-100 as a positive control. The untreated cells were used as a negative control. With the use of Probit-log concentration graphs, the IC_{50} value was calculated.

LDH Release Assay: Following the manufacturer's instructions, the LDH test was carried out using the CytoSelectTM LDH Cytotoxicity Assay Kit (Cell BioLabs, San Diego, CA). Briefly, after the above-mentioned treatments, 90 µl of the supernatant from the cells were transferred to a fresh plate, and 10 L of the reaction mixture were applied to each well. The reaction was incubated for 30 minutes at room temperature in the dark. Ultimately, a microplate reader was used to detect the optical density at a wavelength of 450 nm. (Synergy-HT; BioTek Winooski, VT, USA). As a positive control, cells were treated with 0.1% (w/v) Triton X-100. The cells without treatment served as negative control.

Oxidative analysis: TAC assay and TOS assay were carried out according to provider's manual. Briefly, the cells were cultured in 96-well plate and treated with 5-HTP in a concentration of 95.23 mg/ml for 48 h. At the end of incubation, commercially available TAC and TOS assay kits (Rel Assay Diagnostics®, Turkey) were used according the manufacturer's instructions to measure antioxidative/oxidative capacity of 5-HTP. Ascorbic acid (10 µM) and hydrogen peroxide (25 µM) from Sigma-Aldrich Company were preferred as positive control treatments in determining TAC and TOS levels, respectively.

Statistical Analysis: Statistical analysis was conducted using SPSS® 21.0 program. The results are given as mean ± standard deviation. Duncan's test was used as a post-hoc followed by one-way analysis of variance (ANOVA). $p < 0.05$ was set as the minimal level of significance.

Results

Two different cell viability assays were carried out to measure the anti-proliferative effect of 5-HTP and L-tryptophan on the human prostate cancer cells in order to obtain more reliable data. Markers of energy metabolism of cell is measured by MTT reduction assay and loss of cell membrane integrity was determined by LDH release assay. The cytotoxicity assays demonstrated that 5-HTP led to decreased cell growth depending on dose ($p<0.05$) (Figure 1).

As a result of the study, IC_{50} values of 5-HTP was determined as 95.23 mg/ml for PC3 and 108.58 mg/ml for Du-145 cells. On the other hand, IC_{50} values of L-tryptophan was calculated as 543.67 mg/ml for PC3 and 793.12 mg/ml for Du-145 cells. Also, LDH release assay exhibited similar results with MTT assay (Figure 2).

Since 5-HTP showed highest cytotoxic activity on PC-3 cells, antioxidative capacity was determined using TAC and TOC assays. Treatments of cells with 5-HTP (at IC20 concentration) and positive control agents (as ascorbic acid and hydrogen peroxide) resulted in changes of TAC and TOS levels as compared to untreated cells. 5-HTP considerably slightly expanded TAC level (1.9x fold) on PC3 cells while this concentration did not cause a change in TOS level. (Table 1).

Discussion

One of the most used criteria for cytotoxicity is cell membrane integrity, and many approaches have been established for its evaluation (22). Numerous investigations have employed the release of stable intracellular enzymes like lactate dehydrogenase (LDH)1 as well as the release of a label like 51Cr or calcein (23–26). Other options for assessing general cytotoxicity include tests that evaluate cell metabolic activity. For instance, many tetrazolium compounds have been utilized for this (27,28). In the present study, we assessed MTT and LDH release assay and both results showed a dose-dependent reduction in viability of both prostate cancer cells after 48 h incubation with 5-HTP.

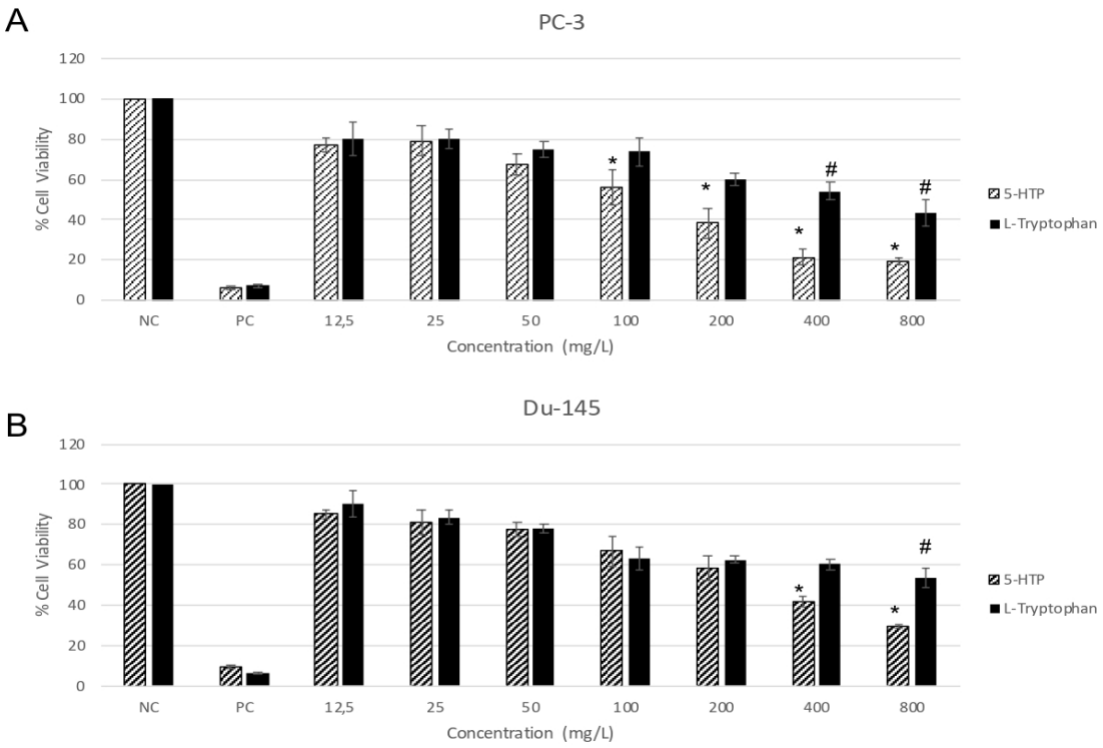

Figure 1. Effects of 5-HTP and L-tryptophan (0-800 mg/ml) on viability of PC-3 (A) and Du-145 (B) cells. Data represented as mean ± SEM (*p <0.05).

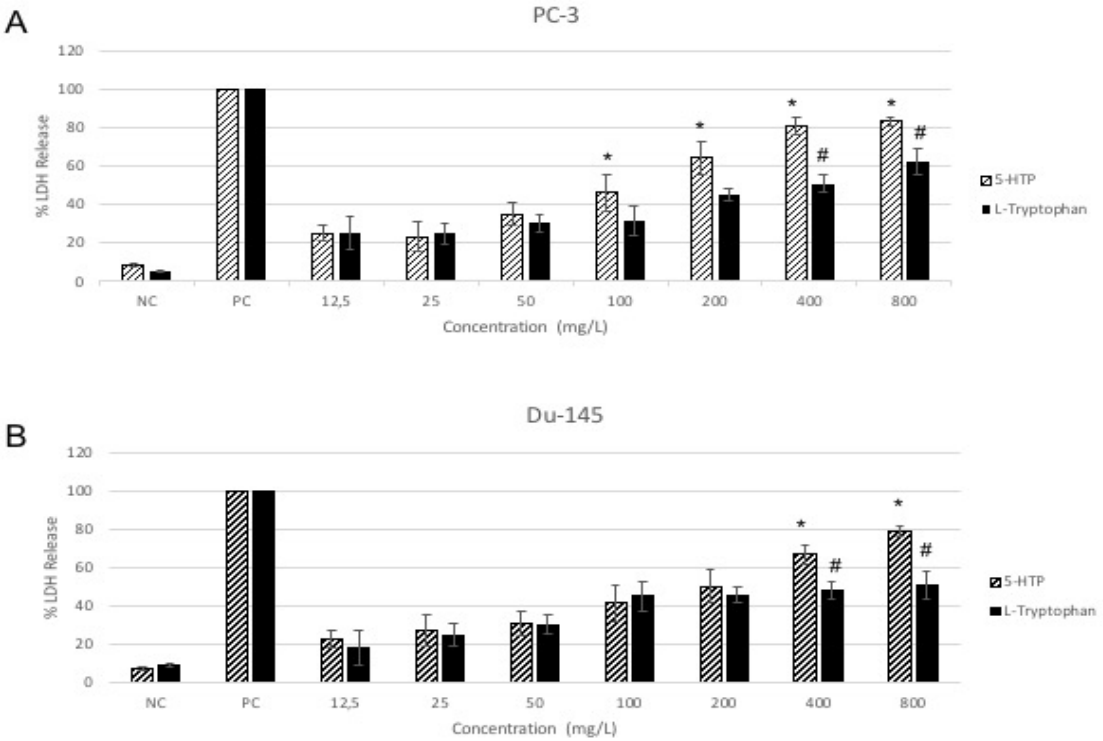

Figure 2. Effects of 5-HTP and L-tryptophan (0-800 mg/ml) on LDH activity of PC-3 (A) and Du-145 (B) cells. Data represented as mean ± SEM (*p <0.05).

Oxidative stress is a factor in the development of a number of diseases and is brought on by the interaction of chemically reactive oxygen species with biomolecules. Angiogenesis, uncontrolled proliferation, apoptosis escape, uncontrolled proliferation, tissue invasion, and metastasis are all associated with oxidative stress at different phases of cancer development (29–31). As a result, substances having antioxidant properties may play a crucial role in chemoprevention by lowering oxidative stress.

Because of their low molecular weight, simple absorption, and great action, researchers have recently focused a lot of attention on antioxidative peptides. It has been observed that several amino acids and their derivatives, including cysteine, histidine, tryptophan, lysine, arginine, leucine, valine, and 5-hydroxytryptophan, have antioxidant properties. (32–35). A naturally occurring amino acid containing amino and hydroxy groups, 5-hydroxytryptophan serves as a metabolic key component for the production of the neurotransmitters melatonin and serotonin. The powerful ability of melatonin to scavenge free radicals is influenced by 5-hydroxytryptophan. (36,37). Various tissues have been shown to be protected against oxidative stress by indoles, both natural and synthetic, mostly by scavenging harmful reactive oxygen species (37–39). Several researches have discovered that substituted indoles have antioxidant properties (40–42).

Notably, we found a significant reduction in TOS level and an increased TAC level in 5-HTP treated cells. These results support the antiproliferative potential of 5-HTP as a chemotherapeutic agent in cancer treatment. Collectively, it can be suggested that 5-HTP may have a good potential for anticancer activity in prostate cancer via decreasing oxidative stress and promoting cell death. However, further investigations are still needed to understand the precise mechanism of the anticarcinogenic effect of 5-HTP in prostate cancer.

Funding: This study is supported with project by Erzurum Technical University, Scientific Research Project Support Coordinator (Project number: 2019/06).

Declaration of Interest: No potential conflict of interest relevant to this article was reported.

Authors' Contributions: ÖÖT, NY, TG and AT contributed to the study conception, design and laboratory work. Writing the article (OOT, NY). All authors read and approved the final manuscript. ÖÖT; Özlem Özdemir Tozlu, NY; Nursena Yüksel, TG: Tuğba Gezmiş, AT; Arzugül Tanas.

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Investigation of cytotoxic and genotoxic effects of olive leaf extract on colon cancer cells and normal cell lines

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Cite: *Öztürk E, Çalık F, Ulusoy D. Investigation of cytotoxic and genotoxic effects of olive leaf extract on colon cancer cells and normal cell lines. Eurasian Mol Biochem Sci 2022;1(2): 26-31.*

Received: 19 September 2022, Accepted: 22 October 2022 *DOI. 10.54672/ejmbs.2022.11*

Abstract

Colon cancer is a type of cancer that occurs when cells in the mucous layer membrane that surrounds the inner surface of the large intestine sections multiply unevenly. This study aimed to investigate olive leaf extract's cytotoxic and genotoxic effects on HTC-116 colon cancer cell lines and HDF healthy cell lines. In the study, olive leaf extract was prepared in methanol. The passaged HTC-116 and HDF cell lines were then incubated in Dulbecco's Modified Eagle's Medium (DMEM). Olive leaf extract extracted in methanol was applied to these cell lines at 3, 10, 20, 50, and 80 µg/ml and left for incubation for 24 hours. Then, the cytotoxic effect was determined by MTT analysis. The genotoxic effect of olive leaf extracts applied to HTC-116 and HDF cell lines after a 24-hour incubation period was determined by Hoechst stain. DNA damage was visualized with a DAPI filter on a camera-attached trinocular fluorescence microscope 30 minutes after staining with Hoechst. The MTT analysis revealed that the cytotoxic effects of olive leaf extract applied to the HTC-116 cell line were close to each group after the 24-hour incubation period. As a result of the examination of the genotoxic activity with Hoechst DNA staining in colon cancer cells (HCT-116), olive leaf extract at various concentrations treated to the cell line had a minor genotoxic effect at low doses (3, 10, and 20 µg/ml), while at high concentrations (50 and 80 µg). /ml), the genotoxic effects of the applications were determined. In examining the genotoxic impact of Hoechst DNA staining in the HDF cell line, no genotoxic effect was observed at low doses (3, 10, and 20 µg/ml) of olive leaf extract applied to the cell line at various doses. The little genotoxic effects of high doses (50 and 80 µg/ml) were also observed. Olive leaf contains phenolic substances which inhibit cell proliferation and DNA damage in colon cancer cells and has no toxic effects on normal cells.

Keywords: Olive leaf extract, colon cancer, cytotoxicity, genotoxicity

Introduction

Cancer is a disease that involve the proliferation of cell that have lost their genetic stability and cell cycle control through mutation [1]. More than 14 million new cancer cases were reported worldwide in 2012 [2], raising a need to further develop treatments and preventive strategies. Cancer mainly occurs with aging, and, there are factors other than age that contribute to the development of cancer. There is strong scientific support that the traditional Mediterranean diet (MD) protects against some cancers [3,4]. The effects of MD

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on inflammation have not been fully elucidated [5,6]. MD has shown a protective role in cancer as a whole [6]. However, it is important to understand whether any beneficial effects attributed to MD are due to a particular component of the diet rather than the entire diet. As an example, polyphenol bioactive compounds have shown particular promise.

Olive tree leaves (Olea europaea) (OLE) are an alternative medicine agent widely used in traditional medicine in the Mediterranean region [7]. The bioactive properties of this Leaf have formed a basis for its use as an antioxidant, anti-hypertensive, anti-atherogenic, anti-inflammatory, hypoglycemic and hypocholesterolemic agent [7,8]. OLE components not detected in Olive Fruit oil include flavonoids such as luteolin and apigenin, which show anti-cancer properties [9,10].

In this study, we investigated the effect of olive leaf extract on human colon cancer cells. It was aimed to investigate the cytotoxic and genotoxic effects of olive leaf extract on HT-116 cell line and Human Dermal Fibroblast (HDF) cell lines.

Materials and Methods

Chemicals: HTC-116 and HDF cell lines were taken from YUTAM cancer laboratories and used for examinations. DMEM (Dulbecco's Modified Eagle's Medium), PBS, Penicillin, and L-glutamine were used in cell passages and treatment of oil lead extract. Trypsin EDTA and PBS were used for the passage of cells.

Oil Lead extract preparation: The leaves were ground with a grinder. They were then left to dissolve in methanol, and extraction was completed. Then, a rotary evaporator separated the extract from the solvent (Laborota 4001, Heidolph). The temperature of the water bath in the rotary evaporator was set at 40°C and the rotation frequency at 60 rpm. The pressure in the condenser was adjusted with a vacuum valve to evaporate the solvent faster. The evaporating solvent was concentrated via a condenser. Finally, olive leaf extract was obtained to be used in this study.

Cell viability analysis: The viability of Oil leaf extract treated cells was measured using flow cytometry according to the manufacturer's instructions (Ecotech Biotechnology, Turkiye). HCT-116 and HDF cells were seeded in 96-well plates at 1000 cells per well and treated for 24h at concentrations of 3, 10, 20, 50, and 80 µg/mL of Oil leaf extract diluted in the medium. After incubation for the indicated time, the MTT solution has added to the wells and allowed to be incubated for at least 3 hours in darkness. The cell number and viability were measured by the colorimetric absorbance of cells (Thermo Fischer, USA). The absorbance values were used for the cell viability assay.

Genotoxicity analysis: For preparing Hoechst dye, 20 µl of Hoechst stock dye was added to 50 ml of PBS. For Hoechst staining, the HCT-116 and HDF cell lines were seeded in 24 well-plates and the concentration of Oil lead extract 3, 10, 20, 50, and 80 µg/mL incubated the cells and then, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline at 4 °C for 30 min. The cells were washed with phosphate-buffered solution (PBS), and samples were incubated with 1 mM Hoechst 33258 fluorescent dye (Sigma-Aldrich®, USA) for 5 min at room temperature. Nuclear abnormalities were observed under the fluorescent microscope (Leica® DM IL LED, excitation/emission wavelength = 365/420 nm) on a total of 1000 cells in each well. The scored nuclear alterations (NAs) were divided into the following categories: lobed (L), notched (N), and micronuclei (MN) [11].

Results

Cell viability results: MTT analysis was performed

to observe whether olive leaf extract has cytotoxic effects on both healthy human fibroblast cell line and colon cancer cell line.

Table 1. MTT (Cell viability) values after 24 hours of incubation with olive leaf extract treated to HCT-116 and HDF cell lines

Group	HCT-116		HDF	
	Mean	S.D.	Mean	S.D.
Control	0.174	0.010	0.175	0.050
3 µg/mL	0.154	0.006	0.174	0.022
10 µg/mL	0.157	0.015	0.187	0.077
20 µg/mL	0.153	0.008	0.203	0.006
50 µg/mL	0.162	0.016	0.193	0.032
80 µg/mL	0.160	0.011	0.179	0.036

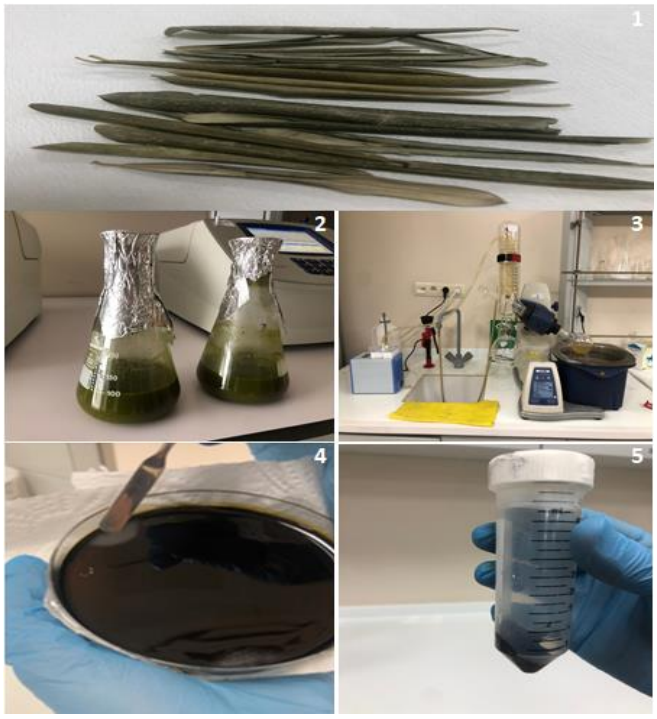

Figure 1. Process of Oil leaf methanol extract preparation; 1: Olive leaves, 2: Homogeneous extract, 3: Evaporator device, 4: Planning by scraping the dried extract, 5: Extract transferred to test tube.

Cytotoxicity was determined after incubation with the olive leaf extract at concentrations of 3, 10, 20, 50 and 80 µg/ml to colon cancer cells (HCT-116) and human dermal fibroblast cells (HDF) for 24 hours. While it was observed that the cytotoxic effects of olive leaf extract concentrations were close to each other in the groups, the most effective cytotoxicity value was found in the group administered at a dose of 20 µg/ml. It was determined that the cytotoxicity of 50 and 80 µg/ml concentrations was less than the other concentrations. After the incubation period, no cytotoxic effect was detected in any of the doses of olive leaf extract on the HDF cells for 24 hours in the MTT analysis. However, it was determined that olive leaf extract at a concentration of 20 µg/ml showed a proliferative effect (Table 1, Fig 2 and 3).

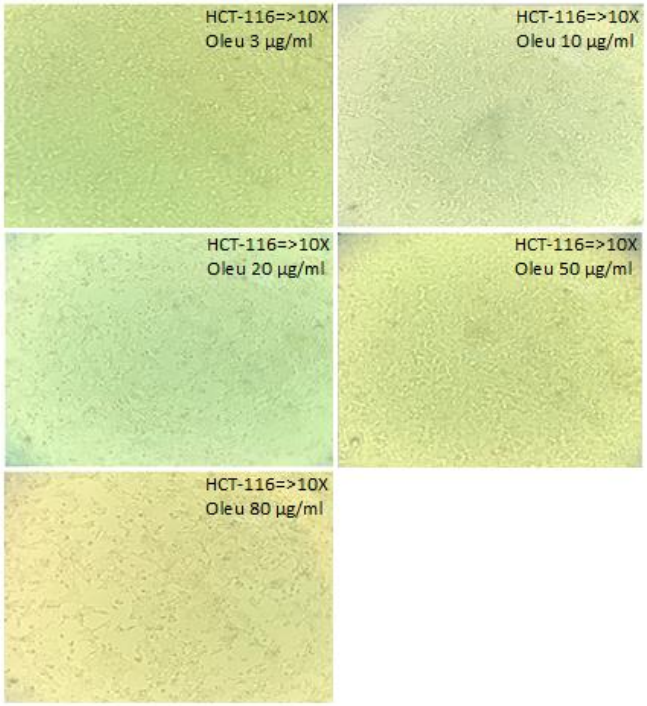

Figure 2. Illustration of inverted Microscope after the application of olive leaf extract at various concentrations to HCT-116 cell lines.

Genotoxic analysis: In evaluating genotoxic activity with Hoechst DNA staining, a small amount of genotoxic effect of olive leaf extract applied to colon cancer cell line at various concentrations was determined at low doses. In contrast, the genotoxic effects of applications at high concentrations (50 and 80 µg/ml) were determined. In the evaluation of genotoxic activity, no genotoxic effect was observed at low doses of olive leaf extract at various doses applied to the human dermal fibroblast cell line. In contrast, genotoxic effects were determined at high doses (50 and 80 µg/ml) (Fig. 4 and 5).

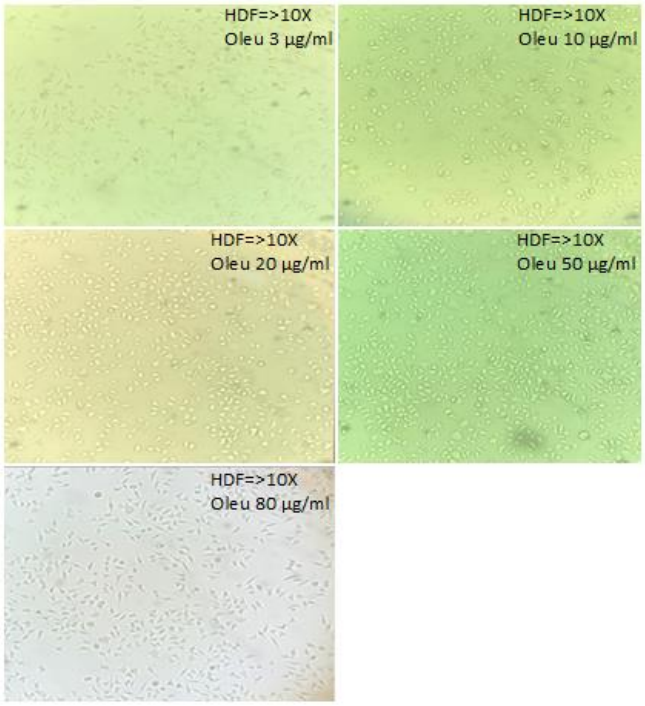

Figure 3. Illustration of inverted Microscope after the application of olive leaf extract at various concentrations to HDF cell lines.

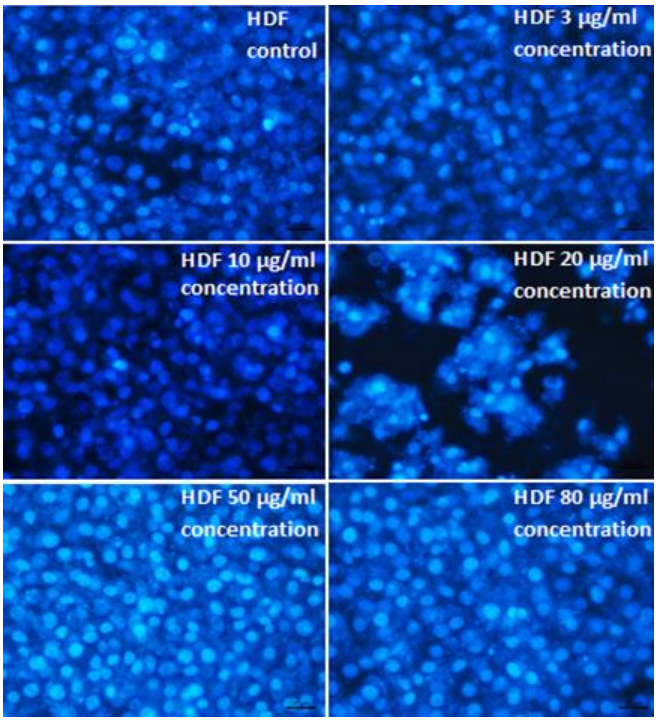

Figure 4. Fluorescent microscope images of the HDF cell line after hoechst dye application

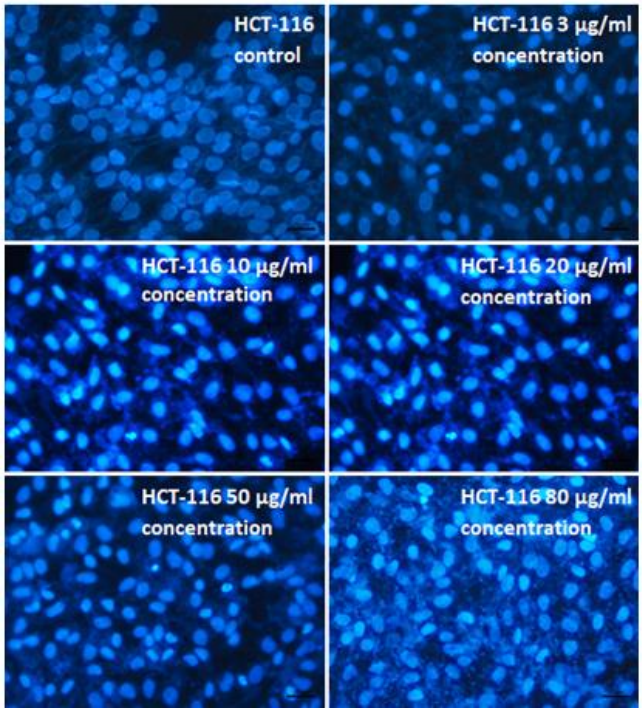

Figure 5. Fluorescent microscope images of the HCT-116 cell line after hoechst dye application

Discussion

Colon cancer is the third most common type of cancer in the world and in Turkey, and ranks third in cancer-related deaths. It is known that most of the colon cancer starts with the abnormal growth of healthy epithelial cells in the mucous layer of the colon or rectum [12]. In this study, it was aimed to investigate the cytotoxic and genotoxic effects of olive leaf extract on colon cancer cells, and additionally, this preliminary study aims to assess the effectiveness of methanol extraction from olive leaves and investigate olive leaf extracts as anticancer agents.

The emergence of colon cancer arises with the interaction of many hereditary, environmental and genetic factors. For these reasons, the susceptibility to mutations and excessive consumption of red meat, insufficient intake of vitamins, bile acids, and mineral intakes, HNPCC is a hereditary type of colon cancer observed at young ages (before the age of 50) and caused an average of 4% of all colon cancer types. This variety is considered genetically predisposed [13].

According to previous research, one of the most important factors in the emergence of colon cancer is the presence of a family history of colon cancer. More than 90% of colon cancer cases were determined accidentally, without any particular symptoms. In the remaining colon cancer cases, it has been determined that there is a high degree of genetic factors. It is known that many such critical genetic factors come together and cause colon cancer [13].

The study showed that olive leaf extract had cytotoxic effects at 20 µg/ml but not cytotoxic at other concentrations on the HCT-116 cell line. Additionally, olive leaf extract was not found to have a toxic effect on HDF cells. Therefore, it was thought that olive leaf extract could accelerate cell death in cancer cell lines. Previous researches were reported that olive leaf extract have cytotoxic effects on many different cancer cells including breast and colon cancers [14,15]. Also, Barbaro et al. [16] reported that the antitumor activity of olive leaf and oleuropein may be associated with reactive oxygens species (ROS).

Antigenotoxic agents often show expected therapeutic effects that may effectively control cancer [17]. A significant number of nuclear abnormalities was determined in the higher concentration of treated colon cancer cells in this study. This result may be explained by olive leaf extract its capacity to act as a potent free radical scavenger [18,19,20,21].

This study's limitations include being limited to one cancer cell line, and the molecular mechanisms underlying activity were not investigated. However, this study provides a platform for further research to evaluate the molecular mechanisms involved in the anticancer activity of the phenolic compounds of olive leaf extract in colon cancer cells.

Declaration of Interest: No potential conflict of interest relevant to this article was reported.

Authors' Contributions: EÖ, FÇ, and DU contributed to the study conception and design. EÖ, FÇ, and DU contribution to laboratory work. Literature research (EÖ and FÇ), Writing the article (EÖ and FÇ). All authors read and approved the final manuscript. EÖ; Emre Öztürk, FÇ; Fatma Çalık, DU; Derya Ulusoy.

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Possible anti-inflammatory role of Probiotics in the treatment of Covid-19 disease

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Cite: *Gelen V, Şengül E. Possible anti-inflammatory role Probiotics in the treatment of Covid-19 (SARS-CoV-2) disease. Eurasian Mol Biochem Sci 2022;1(2): 32-37.*

Received: 05 October 2022, Accepted: 28 October 2022 *DOI. 10.54672/ejmbs.2022.12*

Abstract

Covid-19 is a deadly viral disease prevalent in the world. As a result of the viral disease, a serious inflammatory response develops in the organism. Various research results have reported that the development of this response causes damage to various organs and tissues. Compounds with anti-inflammatory action can reduce or prevent the potential harm caused by this inflammatory response to the organism. Several recent studies suggest that probiotics have powerful anti-inflammatory properties. In conclusion, this study addressed the potential anti-inflammatory effects of probiotics in Covid-19 disease.

Keywords: anti-inflammatory, probiotics, covid-19

Introduction

Covid-19 disease, which causes acute respiratory syndrome and is seen all over the world and is quite deadly, is one of the controversial issues (1). Clinical symptoms of this disease, such as diarrhea, cough, fever, and shortness of breath, are present (2). The clinical signs are mostly asymptomatic or mild (1, 3). In addition, COPD, coagulation disorder, kidney damage, metabolic acidosis, heart failure, or secondary infections can all result from COVID-19 infection (2, 4-12). There is substantial evidence that systemic hyperinflammation contributes to lung and multi-organ failure in Covid-19 patients (1). It was determined that D-dimer, C-reactive protein, IL-6, and procalcitonin levels were increased in the sera of Covid-19 patients. This condition is associated with macrophage activation syndrome and hyperinflammation (3).

Macrophages and monocytes play an important role in the inflammatory responses associated with Covid-19 infection (13). These cells secrete proinflammatory cytokines such as TNF-alpha, IL-1, IL-6 and IL-8 during infection. Excessive cytokine release in Covid-19 disease causes development of multi-organ failure and worsening of the condition (2, 14-17). Consequently, anti-inflammatory agents are critical in the treatment

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of Covid-19 disease to reduce disease severity. Identifying new agents in addition to the currently known therapeutic agents will help develop strategies to combat the pandemic (1). Probiotics are live microorganisms that have been shown in numerous studies to suppress inflammation and protect tissue from the effects of inflammation (14-17). In line with this information, probiotics may be effective in relieving inflammation caused by covid-19. The aim of this study, according to this information, is to explain the effect of probiotic use in addition to existing agents in the treatment of covid-19-induced inflammation.

Morphology of the virus and its attachment to the cell: According to its morphological structure, the Coronavirus is a single-stranded (+) RNA-enclosed virus (18). Photos taken with an electron microscope in 1968 revealed that this virus family resembles the "solar corona," which derives its name from the Latin word "coronavirus" (19).

Four primary structural proteins have been identified in the coronavirus structure. These proteins include: S is a trimeric Spike glycoprotein found on the viral envelope's surface that is required for viral entry into cells. Matrix or membrane protein M is the name given to the second protein. E, the third protein, is a small envelope protein needed for virus collection and release. The nucleocapsid protein, N, is the fourth protein. It forms the symmetrical nucleocapsid by helically attaching to the RNA genome (Figure 1). (20). The virus was thought to enter cells via the ACE2 protein, which is found in abundance in the testis, heart, lung, kidney, and gastrointestinal tract (21). Ang II is converted into Ang 1-7 by the membrane-bound protein ACE2 (22). Several steps are involved in the Covid-19 infection cycle: These are the procedures. 1. Locate and bind to the cell's receptor (S). The second modification affects the structure and proteolysis of the S protein. The third step is fusion with the cellular membrane (23, 24, 25). Figure 1.

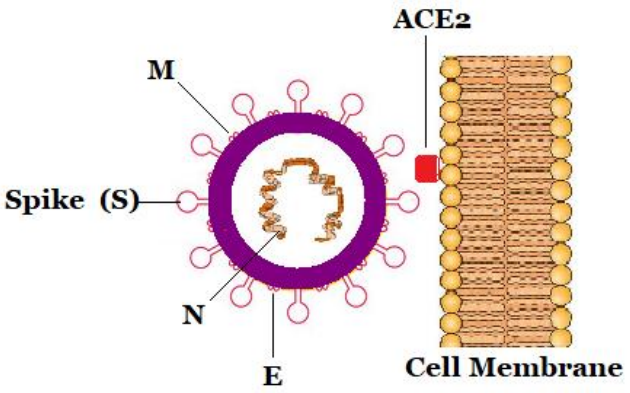

Figure 1. The structure of the coronavirus and its entry point into the cell (26).

The Covid-19 is experiencing a cytokine storm: The cytokine storm caused by Covid-19's inflammatory response may be associated with clinical deterioration and an increased risk of death (27). Blood levels of cytokines increased in Covid-19 patients (28). Furthermore, in severe Covid-19 patients, G-CSF, MCP1, IP10, IL-2, and TNF-alpha levels were found to be quite high (28). The study showed that people who died from severe Covid-19 infection had extremely high IL-6 levels (29).

In one study, a cytokine storm was divided into two stages (30). The absence of immunity is the first stage. A hyperactive immune response characterizes the secondary stage, which appears to be a clinical manifestation of a cytokine storm (31, 32). Low IFN activity and IFN-induced gene down-regulation have been shown to impair type 1 IFN responses as well as IL-6 and TNF-mediated hyper-inflammatory responses (33-38).

Probiotic effect on immune responses: When used correctly, probiotics are living microorganisms that contain a variety of bacteria and yeast strains and have beneficial effects on the host. Leuconostoc, Pediococcus, Lactobacillus, Bifidobacterium, and Enterococcus are all probiotic bacteria (40). Probiotics regulate, and modulate a variety of functions in the intestine, including digestion, metabolism, and brain-intestinal communication (41, 42). Non-toxic

metabolites produced by intestinal microorganisms play important roles (43-45). Probiotics fulfill three roles including metabolic, protective, and trophic (46). Probiotics produce energy by fermenting indigestible foods known as prebiotics, and they have antipathogenic, antiobesity, antidiabetic, anti-inflammatory, anticancer, and angiogenic properties, as well as effects on the brain and central nervous system (47).

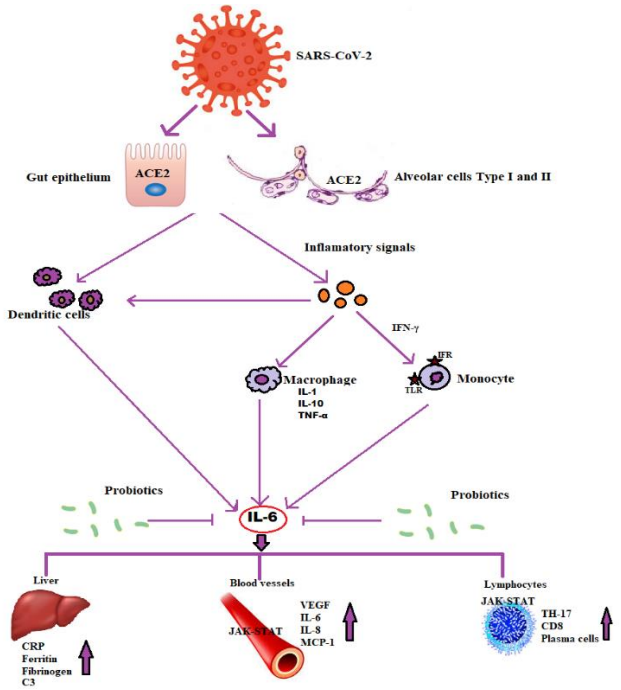

Figure 2. Covid-19 is in the grip of a cytokine storm. Probiotics have anti-inflammatory properties. TNF- stands for tumor necrosis factor alpha; IFN stands for interferon; IL stands for interleukin; and JAK/STAT. CD8 is an abbreviation for cluster of differentiation 8, TH-17 is an abbreviation for T helper 17, and VEGF is an abbreviation for vascular endothelial growth factor. MCP-1 is an abbreviation for monocyte chemoattractant protein-1, and CRP is an abbreviation for C-reactive protein. C3 is an abbreviation for complement component 3, ACE2 is an abbreviation for angiotensin-converting enzyme 2, TLR is an abbreviation for toll-like receptor, and IFR is an abbreviation for interferon (39).

Probiotics have important roles in humoral, cellular and nonspecific immunity. In addition, studies have shown that probiotics also have an effect on the immune barrier (48, 49). It has been reported that probiotics increase peripheral immunoglobin production, stimulate IgA secretion and inhibit the production of proinflammatory cytokines (50, 51). Probiotic bacteria regulate epithelial cell proteasomal activity and it has been determined that they may play a role in the epithelial-derived T cell activation mechanism of the intestine (52, 53). It has been shown that probiotics produce non-living metabolic byproducts such as bacteriocins and organic acids that are resistant to mammalian enzyme systems, non-toxic and non-pathogenic, and can be used as an alternative to antibiotics due to their biological activities and inhibitory properties (54, 55). Probiotics increase antioxidant production (glutathione) and reduce oxidative stress, according to some studies. Probiotic microorganisms inhibit lipid peroxidation and reduce STZ-induced oxidative damage in rat pancreatic tissues (56, 57). Various studies have revealed the basic molecular mechanisms of probiotics, such as IgA secretion, cytokine production, antibacterial agent production, tight junction enhancement against intercellular bacterial invasion, and competition for enterocyte adhesion with novel pathogenic microorganisms. The immunomodulatory effect of probiotics is closely related to the release of cytokines from immune cells such as lymphocytes, granulocytes, and macrophages (58).

Probiotic strains influence the gut barrier by inducing IgA production in B cells. In vitro, probiotics have been shown to influence cytokine production by antigen-presenting cells (APCs), which initiate adaptive responses in enterocyte cells. Cytokines also help the immune system fight of fungi, viruses, bacteria, and other pathogens. Immunostimulatory probiotics fight inflammation and cancer cells by increasing IL-12 production, which activates NK cells and promotes the proliferation of Th1 cells. Probiotics can also aid in the treatment of allergies by balancing the Th1 and Th2 immune systems. Immunomodulatory probiotics, on the other hand, have been shown to decrease allergies, inflammatory responses, and IBD by increasing IL-10

and Treg cell production (59). Probiotics have anti-inflammatory properties. Probiotics boost IL-10 while suppressing IL-12. (60). Probiotics either activate the immune system by increasing levels of IL-12, IL-1, and TNF-α, or they act as an anti-inflammatory by increasing levels of IL-10 and TGF-β (61). T helper cells contribute to immune responses. Proinflammatory cytokines are produced by Th1/Th17 cells. Treg cells inhibit T cell functions like Th1, Th2, and Th17. IFN and IL-10 levels can be reduced in L. plantarum and B. infantis (62, 63). Probiotic mixtures can also reduce the production of proinflammatory cytokines such as IL-17, IFN, and TNF-α while increasing the production of IL-10 and/or Treg cells (64). Morbidity increases during acute lung infection, according to in vivo studies on mice that do not contain microorganisms (65). Another study discovered a link between Mycobacterium tuberculosis infection severity and the intestinal microbiota (66). Furthermore, as a result of previous research, we discovered that probiotic application suppressed the increase in cytokines, which increased as a result of inflammation caused by various toxic agents in rats (14-17).

CONCLUSION

As a result, a more effective treatment method for the highly contagious and lethal coronavirus epidemic has yet to be discovered. This situation motivates researchers to seek alternatives to human coronavirus infections. According to various studies, probiotics play an important role in reducing inflammation in various tissues. Coronavirus has been shown to cause severe inflammation and death after tissue damage in a variety of tissues. Coronavirus has been shown to cause severe inflammation and death after tissue damage in a variety of tissues. In this context, we believe that the probiotics mentioned can be used as an alternative to the current anti-coronavirus agents.

Declaration of Interest: No potential conflict of interest relevant to this article was reported.

Authors' Contributions: VG and EŞ contributed to the study conception and design. Literature research (VG and EŞ), Writing the article (VG). All authors read and approved the final manuscript.

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2- Manuscripts can only be submitted through the journal's online manuscript submission and evaluation system, available at **https://dergi.erzurum.edu.tr/ejmbs/about/submissions**. During the submission process, the authors should upload the manuscript's figures to the online manuscript submission system. The **Copyright Transfer Agreement Form** is signed by all the authors and must upload the submission system.

3- The authors should indicate the institute's name approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

Manuscripts Types

Original (Full-Article) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations, and experiments. Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, acknowledgment, Declaration of Interest, and references, and it should not exceed 12 pages, including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. The Abstract should contain 250 words. **The template of the Full article main text is here.**

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be an original full-length article. They should be prepared in the format of the original full-length article, but the Abstract should not exceed 100 words, the reference numbers should not exceed 15, and the text length should be no longer than 6 pages in total.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles**.**

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits. The length of the text should be no longer than three pages in total. The page limit includes tables and illustrations.

Reviews must include criteria for consideration: a critical overview of the field, with a strong focus on current challenges and the bottlenecks to future major advances. Please make sure to emphasize the molecular mechanistic aspects where possible Otherwise, please feel free to decide the exact scope of your review and the depth of coverage, keeping in mind that the overall message should be accessible to the journal's broad readership. Also, the review must represent a fair and unbiased coverage of the most recent advances and relevant approaches and research groups working in the field and accommodate different points of view. Finally, we would encourage you to include a 'perspectives' paragraph at the end of your review that proposes the direction you personally consider the field should take and the prospects for useful applications you might expect to see in the near future and beyond. The title and summary of this manuscript should be prepared as described for the full-length original articles, and the remaining sections should be followed by abstract, introduction, text (with appropriate titles), conclusion, and references.

Title page

A separate title page should be submitted with all submissions, and this page should include (**Please use Template of Title Page**):

- The full title of the manuscript as well as a short title (running head) of no more than 60 characters,
- Name(s), affiliations, and ORCID IDs of the author(s),
- Grant information and detailed information on the other sources of support,
- Name, address, telephone (including the mobile phone number), and email address of the corresponding author.
- Authors' Contributions (The contributions of all authors should be described on the title page of the manuscript using the CRediT Taxonomy of author roles).

Figures and figure caption

You should not attach figure files in the manuscript or you should upload your figure files to our e-submission system while submitting your paper.

Figures should be submitted in Tiff or JPEJ file format, and they will be automatically attached at the end of the manuscript in PDF. Number figures as Figure 1, Figure 2 ... in order of citation in the figure legend. An individual should not be recognizable in the photographs unless written consent of the subject has been obtained and is provided at the time of submission. Scales should be presented as a bar in the picture or as a magnification remark in the legend.

Except for especially complicated drawings that show large amounts of data, all figures are published at one page or one column width; when the figures are reduced to the size of a single column or of the single page width, the smallest parts of the figure must be legible.

It is recommended to size original figure widths to 4 inches wide. The minimum requirements for digital resolution are:

- 900 DPI/PPI for black and white images, such as line drawings or graphs.
- 300 DPI/PPI for picture-only photographs.
- 600 DPI/PPI for photographs containing pictures and line elements, i.e., text labels, thin lines, arrows.

The abbreviations for a minute, hour, and day are min, hr, and d, respectively, and the abbreviations for a week, month, and year are wk, mo, and yr, respectively. These abbreviations are used in tables, figures, and virgule constructions.

Summary data (e.g., means, odds ratios) should be reported using data markers for point estimates, not bars, and should include error bars indicating measures of uncertainty (e.g., SDs, 95% CIs). Actual values (not log-transformed values) of relative data (for example, odds ratios, hazard ratios) should be plotted on log scales.

The figures should be at least 300 dpi resolution in your Manuscript Word file, and place a caption below each figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used. This is designed to help the reviewers evaluate your work more efficiently.

Tables and table caption

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

Highlights (Optional)

Highlights are optional for this journal. Specifications: include 3 to 5 bullet points (max. 85 characters per bullet point including spaces); only the core results of the paper should be covered. The first bullet point should state the background or context of the question. One to three bullet points should describe the principal results. The last bullet point should conclude with a clear description of the conceptual advance and significance of the work. Highlights should be submitted as a separate file in the Online submission system when uploading files. Highlights will be also in the article PDF file. **An example of Highlights Template is here**.

Abstract

The abstract must be concise (limit of 250 words) and factual. It should convey the concept and the importance of the paper to non-specialist readers. The Abstract should briefly state the background of the question and the principal results and conclude with a clear description of the conceptual advance and significance of the work. Detailed descriptions of the study or the findings should not be included in the abstract. An abstract is required for all papers. An abstract is often presented separately from the article, so it must be able to stand alone. Also, non-standard or uncommon abbreviations should be avoided, but if essential, they must be defined at their first mention in the abstract itself.

Keywords

Authors should supply five keywords after the Abstract. Keywords should not be words from the title.

Abbreviations

Abbreviations should be avoided as much as possible. When they are used, the full expression of the abbreviations in parentheses following the abbreviated word should be given at the first use.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Materials and Methods (primary section heading)

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Subsection headings should be structured hierarchically as follows:

Secondary subsection heading

Tertiary subsection heading

Quaternary subsection heading:

Ethical approval of studies and informed consent

For all manuscripts reporting data from studies involving human participants or animals, formal review and approval, or formal review and waiver, by an appropriate institutional review board or ethics committee is required and should be described in the Methods section.

General text style

Generic and brand name of medicine or chemical.

For medicine, use generic names. If a brand name should be used, insert it in parentheses after the generic name.

Statistical analysis

Standard deviation and the standard error should be described in the format of mean±SD and mean±SE, respectively. *P*-values should be described as $P<0.05$ or $P=0.003$.

Units

Scientific units should be followed by the International System of Units (http://www.bipm.org/en/measurement-units). Unit for volume is 'L', instead of 'l' to avoid confusion; Unit for blood pressure is mmHg; Temperature is expressed in Celsius and concentration in M, mM, and μM.

Results

Results should be clear and concise.

Tables and figures should be indicated in the main text as follows:

(Table 1), (Tables 1 and 2), (Tables 1-3), (Figure 1A and B), (Figure 1A-C), (Figures 1 and 2), (Figures 1-3), (Figures 1A and 3B), (Table 1, Figure 2).

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Acknowledgments

Collate acknowledgments in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title, or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proofreading the article, etc.).

Declaration of Interest

The statement must be provided as a separate section (titled 'Declaration of interest') at the end of the main text, before the 'References' section.

Reference style

References should be listed at the end of the manuscript. They should be listed in the order in which they appear in the text, tables, figure legends, and numbered sequentially. When cited in the text, reference numbers should be given in parenthesis. Only papers that have been published or accepted should be cited in the reference list. If there are six or fewer authors in a reference, then all the names of the authors should be listed. If the number of authors is more than six, list the initial six authors and then abbreviate the rest of the authors with 'et al'., the title of the article, journal title (abbreviated), year of the article, the volume number, and first and last pages should be cited.

Examples:

1- Published articles:

(Author. Title. Journal name Year:Vol;Page.)

1- Kara A, Akman S, Ozkanlar S, Tozoglu U, Kalkan Y, Canakci, CF, et al. Immune modulatory and antioxidant effects of melatonin in experimental periodontitis in rats. Free Radic Biol Med 2013;55:21–26.

2- Kim CS, Choi SH, Chai JK, Cho KS, Moon IS, Wikesjo UM, et al. Periodontal repair in surgically created intrabony defects in dogs: influence of the number of bone walls on healing response. J Periodontol 2004;75:229-35.

Book

(Author. Book title. Edition. Place: Publisher; Year.)

Newman MG, Takei HT, Klokkevold PR, Carranza FA. Carranza's clinical periodontology. 10th ed. St. Louis: Saunder Elsevier; 2006.

Book chapter

(Chapter author. Chapter title. In: editor(s). Book Title. Edition. Place: Publisher; Year. Chapter page.)

1- Hanks SK, Hunter T. The eukaryotic protein kinase superfamily. In the Protein Kinase FactsBook: Protein-Serine Kinases (Hardie, G. & Hanks, S., eds), London, Academic Press, 1995. p.747.

2- Carranza FA, Takei HH. Clinical Diagnosis. In: Newman MG, Takei HT, Klokkevold PR, Carranza FA. Carranza's clinical periodontology. 10th ed. St. Louis: Saunder Elsevier; 2006. p.540-60.

Web site

(Title [Internet]. Place: Publisher; Copyright year [cited Year Month Date]. Available from: URL.)

American Cancer Society. Cancer Reference Information [Internet]. Atlanta (GA): American Cancer Society; c2010 [cited 2010 Jun 20]. Available from: http://www.cancer.org/docroot/CRI/CRI_0.asp.

Other types of references not described below should follow The NLM Style Guide for Authors, Editors, and Publishers (http://www.nlm.nih.gov/citingmedicine.).

Reference management software

These include all products that support Citation Style Language styles, such as EndNote. **The Endnote reference style link is here.**

Publication fee

There are no fees for page charges or color figures.

Offprints

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Supplemental data

The acceptance of supplemental material is at the Editor's discretion. Supplemental information must be submitted with the manuscript for review by the editor and referees. Manuscripts must be complete and stand-alone. Supplemental material should complement the printed paper and may include figures and figure legends, tables, supporting data, sequence alignments, primers, derivation of equations, and videos. The availability of supplemental information will be indicated in the printed paper and the supplemental data will be directly linked to the online version of the paper. Reference to the supplemental information may be made at appropriate places in the text.

With the exception of videos, the supplemental information must be submitted electronically in the form of a single PDF file. Very large tabulations of supporting data may be submitted as Microsoft Excel files.

Submission checklist

The following list will be helpful during the final checking of an article before finalizing your submission. Ensure that submission files contain, in the following order:

- Title page
- Highlights (optional)
- Abstract (and Keywords)
- Manuscript main text
- Declaration of authors contribution and funding information in the acknowledgment
- Declaration of interest statement
- References
- Figure captions
- Tables
- Figures
- Copyright Transfer Agreement Form
- Similarity report
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Submission of Manuscript

Authors are requested to submit their papers electronically by an e-submission system which can be accessed on the official website of Eurasian Mol Biochem Sci (**https://dergi.erzurum.edu.tr/ejmbs/about/submissions**) as well. After creating an account, authors can proceed with the submission process as arranged. Before submission, the instructions described in the Eurasian Mol Biochem Sci manuscript template files should be carefully reviewed in formatting the papers. The title page, manuscript main text (including tables and figures), and copyright form should be included in the submission file package.

Revisions

When submitting a revised version of a paper, the author must submit a detailed "Response to the reviewers" that states point by point how each issue raised by the reviewers has been covered and where it can be found (each reviewer's comment, followed by the author's reply and line numbers where the changes have been made) as well as an annotated copy of the main document. Revised manuscripts must be submitted within 30 days from the date of the decision letter. If the revised version of the manuscript is not submitted within the allocated time, the revision option may be canceled. If the submitting author(s) believe that additional time is required, they should request this extension before the initial 30-day period is over.

Accepted manuscripts are copy-edited for grammar, punctuation, and format by professional language editors. Once the publication process of a manuscript is completed, it is published online on the journal's

webpage in its scheduled issue. A PDF proof of the accepted manuscript is sent to the corresponding author and their publication approval is requested within 2 days of their receipt of the proof.