International Journal of Nature and Life Sciences IJNLS

https://dergipark.org.tr/tr/pub/ijnls

e-ISSN: 2602-2397 https://doi.org/10.47947/ijnls.1477018

Research Article

An In Vitro Study: Assessment of Gene Expression Changes Induced by Nanoparticles Synthesized from *Cladonia subulata* Lichen on Colon Cell Lines

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Received: May 2, 2024

Accepted: May 23, 2024

Online Published: June 1, 2024

Citation:

Ata, M. S., Avuloğlu Yılmaz, E., Polatcı, Ş. & Bölülbaşı, E. (2024). An in vitro study: assessment of gene expression changes induced by nanoparticles synthesized from *Cladonia subulata* Lichen on colon cell linesbe added by editorial staff during production. International Journal of Nature and Life Sciences, 8 (1), 23-35.

Abstract: Lichens are among the living organisms used for the biological synthesis of nanoparticles. Lichens are typically organisms where algae and fungi exist symbiotically. These organisms possess a wide array of biological components and are particularly rich in secondary metabolites. These characteristics give them an advantage in NPs synthesis. The use of microorganisms and plant sources in biological synthesis provides a less toxic and more environmentally friendly alternative compared to chemical methods. This study examined the effects of copper-based NPs obtained through biological synthesis from *Cladonia subulata* (L.) lichen on colon cancer cells from a molecular biology perspective. DLD-1 (colon cancer) and CCD18-Co (healthy colon) cell line were treated with concentrations of NPs ranging from 3.91 to 500 µg/ml for 24 hours, based on the evaluation of MTT test results, and changes in the expression levels of the *CYP1A1* and *BCL-2* genes involved in the cancer pathway were detected. In DLD-1 cells, a significant increase in the expression levels of the BCL-2 and CYP1A1 genes was observed following the application of Cu-NPs. This increase is believed to support the anti-cancer properties of Cu-NPs by affecting uncontrolled cell proliferation. Similarly, Cu-NPs increased the expression levels of the *BCL-2* and *CYP1A1* genes in CCD18-Co cells. The highest expression levels were observed at 125 µg/ml in both cell lines. These results suggest that Cu-NPs may also exhibit anti-cancer effects in healthy cells. The results support the evaluation of NPs obtained through biological synthesis as a potential strategy in cancer treatment.

Keywords: *Cladonia subulata*, Lichen, Nanoparticle, DLD-1, CCD18-Co.

1. Introduction

Nanotechnology is a discipline dealing with the control and manipulation of materials at the atomic and molecular levels, which has made significant advancements in science and engineering in recent years. The examination of materials at the nanometer scale, i.e., at dimensions of one nanometer, leads to the emergence of different and sometimes unexpected properties compared to traditional materials. This renders nanotechnology highly diverse and innovative (Klaus-Joerger et al., 2001; Ahmad et al., 2003). While the efficacy

of nanoparticles often related to their size, shape also influence nanoparticle properties.There has been a trend toward an increased preference for nanoparticles due to their high surface area and greater efficiency. In particular, the introduction of nanotechnology into the medical field has enabled various applications such as the use of nanoparticles in biosensors, drug delivery and selective targeting (Shende et al., 2018).

Owing to their unique electronic, magnetic, catalytic and optical properties, metal nanoparticles find new and exciting applications across a wide range of fields, from biomedical sciences to electronics and optics. Among metal nanoparticles, silver (Ag) and gold (Au) nanoparticles stand out due to their antibacterial properties and potential uses in biomedical applications. Silver nanoparticles are utilized in a wide range of applications, from textile products to wound dressings, due to their high antibacterial efficacy (Morones et al., 2005; Sondi et al., 2004), while gold nanoparticles are evaluated in sensitive biomedical applications used in cancer therapy and diagnosis (El-Sayed, 2001; Alivisatos, 2004). Copper nanoparticles (CuNPs) offer economic advantages compared to silver and gold nanoparticles and have potential applications in electronics and thermal management. The antimicrobial properties of copper may also provide an alternative solution to silver, potentially leading to increased use of copper nanoparticles in various industrial and medical applications (Lee et al., 2016; Mittal et al., 2013). However, research on the biological synthesis and applications of copper nanoparticles is still in its early stages, necessitating further investigation in this area (Iravani, 2011; Dahoumane et al., 2017). Therefore, biological synthesis methods have gained importance along with traditional chemical and physical methods (Thakkar et al., 2010; Singh et al., 2016).

Biological synthesis methods enable sustainable production of metal nanoparticles by offering environmentally friendly and energy-efficient production processes. These methods involve the utilization of microorganisms and plant sources, providing a less toxic and eco-friendly alternative compared to chemical methods (Shah et al., 2015; Park et al., 2011). The production of NPs from plant and microbial sources has recently become the focus of scientists because of the their eco-friendliness and simplicity of the production process compared to other methods (Panpatte et al., 2016). In recent years, many studies have been conducted on the biological synthesis of metal nanoparticles using plant extracts and microorganisms by green chemistry principles (Kumar and Yadav, 2009; Narayanan and Sakthivel, 2010; Koca and Duman, 2019).

Lichens are among the living groups used in the biological synthesis of nanoparticles. Lichens are typically combinations of algae and fungi that are often in a symbiotic relationship with fungi. These organisms possess a wide variety of biological components and are particularly rich in secondary metabolites. These characteristics are advantageous for the synthesis of nanoparticles. Specifically, lichen extracts enable the synthesis of nanoparticles of various sizes and shapes by reacting with metal salts (Brodo et al., 2001).

Cladonia (L.) is one of the largest genera of lichens, comprising approximately 475 species exhibiting various morphologies (Pino-Bodas and Stenroos, 2021). *Cladonia subulata* (L.) is a species within the *Cladonia* genus (Tucker, 2002). Discrimination of *Cladonia subulata*, which possesses numerous morphological characteristics, is based on podetia and lichen acids (Dolnik et al., 2010). A recent study found that *Cladonia subulata* exhibited cytotoxic activity potent enough to potentially provide cancer treatment (Hawryl et al., 2020) (Figure1).

Figure 1. The general appearance of *Cladonia subulata* (L.) (Nash et al., 2002).

Advancements in technology, coupled with the widespread use of numerous chemical agents in technological products, have led to an increase in the incidence of fatal diseases such as cancer. Particularly, colorectal cancer is the third most common cancer worldwide and the second leading cause of cancer-related death (Wong et al., 2022). Colorectal cancer originates from abnormal growth of colonic epithelial cells and culminates in a dangerous tumor. Similar to other cancer types, early diagnosis in colorectal cancer, followed by prompt treatment, can extend survival rates, thus indicating successful intervention. Rapidly evolving technology has contributed to favorable outcomes in the early detection and treatment of cancer (Esmeeta et al., 2022).

Some methods or analyses commonly used in the early diagnosis and treatment of cancer can detect potential DNA damage. These include the comet assay and micronucleus analysis. Additionally, gene expression analysis can be used to understand the mechanisms underlying the potential genotoxic effects of chemicals and to identify early molecular markers associated with carcinogenicity (Bolukbasi et al., 2023). Therefore, real-time reverse transcription (RT) polymerase chain reaction (PCR) has become the preferred method for determining the highly efficient and accurate expression profiles of selected genes (Hwang and Lee, 2012; Bolukbasi, 2021; 2022).

Changes in the expression of cancer-associated genes indicate the potential genotoxic effects of a substance on these genes. Therefore, evaluating the genotoxicity profiles of these chemicals in terms of their genes may be beneficial. A positive result in genotoxicity tests occurs when a compound or its metabolites interact directly (covalent binding) or indirectly (microtubule proteins) with DNA. This result may involve a permanent alteration in the DNA base sequence and/or the complete loss of part of the chromosome structure. If such changes affect specific genes/genomic regions and combine with other conditions in the multistep process of cancer development, they are generally associated with an increased tumor incidence (Cartus and Schrenk, 2017).

The human *CYP1A1* (*cytochrome P450*) gene is a significant member of the *CYP1A* family, which plays a role in converting procarcinogens (polycyclic aromatic hydrocarbons and aromatic amines) into reactive metabolites. Cytochrome P450 enzymes are involved in the oxidative metabolism of endogenous compounds (such as steroids, fatty acids, leukotrienes, and prostaglandins) and exogenous compounds (such as drugs), as well as in the metabolism of carcinogens and other environmental pollutants (Masson et al., 2005; Bolukbasi et al., 2023). The *BCL-2* (*B-Cell Lymphoma 2*) gene encodes the BCL-2 protein family, which controls cell death through direct binding interactions. These proteins regulate the permeability of the outer mitochondrial membrane (MOMP), leading to the subsequent release of intermembrane space proteins following caspase activation and apoptosis. These proteins are regulators of apoptosis, but they also have other functions (Warren et al., 2019; Bolukbasi et al., 2023).

In this study, the cytotoxic effect of Cu-flower-shaped nanoparticles derived from *Cladonia subulata* lichen on colon cancer cells (DLD-1) and healthy colon cells (CCD18-Co) was determined using a MTT assay. Subsequently, alterations in the expression levels of *CYP1A1* and *BCL-2* genes in DLD-1 and CCD18-Co cell lines were investigated based on the data obtained from the MTT assay and the application of the synthesized Cu-flower-shaped nanoparticles at doses determined for DLD-1 and CCD18-Co cell lines.

2. Materials and Methods

2.1. Obtaining lichen extract for the synthesis of nano-materials

Specimens of *Cladonia subulata* lichen were obtained from Erciyes University Herbarium, Kayseri, Turkey (ERCH). The *C. subulata* lichen was washed and allowed to dry. After drying, 10 g of *C. subulata* lichen was weighed and soaked in 100 ml distilled water at 85°C for 1 hour. The mixture was then centrifuged at 5000 rpm for 5 min. Subsequently, the filtered extracts using Whatman No. 1 filter paper were preserved for use in the synthesis of flower-shaped hybrid nanostructures.

2.2. Synthesis of flower-shaped hybrid nanostructures

To determine the optimized synthesis conditions for the synthesis of flower-shaped hybrid nanostructures, lichen extracts were taken in different volumes (0.5 ml, 1.5 ml) and subjected to vortexing with Cu ($8x10^{-4}$ M, 0.35 ml) in 10 mM PBS buffer prepared at different pH levels in falcon tubes. After incubation of the resulting mixture in a dark environment (3 days, 4 °C), the blue-colored solutions formed at the bottom of the

falcon tubes were centrifuged (10000 rpm, 10 min). Subsequently, they were washed with distilled water and dried overnight in an oven at 70 °C (Koca and Duman, 2019).

2.3. Cell culture and MTT assay

Colon cancer cells (DLD-1) cultivated at the Central Research Laboratory of Amasya University were used in this study. The cells were examined under an inverted light microscope and passaged in sufficient amounts. They were prepared for analysis in 75 cm^2 flasks. The adherent cells on the flask surface were washed twice with phosphate-buffered saline (PBS) and then removed with 1X Trypsin/EDTA solution. After neutralization with the culture medium, they were transferred to falcon tubes and centrifuged at 1600 rpm for 10 min. The supernatant was discarded, and 1 ml of culture medium was added to the pellet to prepare for counting. The prepared cell suspension was stained for 10 s on a Thoma slide. Cell counting was performed using a light microscope. Cells were diluted with culture medium to a concentration of $5x10^3$ cells/100 µl of medium per well, and then seeded onto 96-well plates with flat bottom, single-use, and sterile lids (used for analysis). The cells were then incubated at 37°C in a 5 % CO_2 atmosphere. After incubation, the cell culture medium was replaced with a medium containing Cu NP solution at concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81 and 3.91 µg/ml and incubated for 24 h. After 24 h of incubation, the MTT solution (prepared by dissolving MTT in PBS to a concentration of 5 mg/ml and filtering) was added to each well and incubated for 3 h. The culture medium was then completely removed and 100 µl of DMSO was added to each well. The plates were then shaken in the dark for 15 min. to dissolve the formazan crystals (Ghasemi et al., 2021). Cell viability was measured using the following formula in Microsoft Excel: Viability (%) = (average experimental optical density (OD) value / average control OD value) x 100. For the MTT assay results, one-way ANOVA (Tukey) was performed using SPSS 25.0 (IBM SPSS, Inc., Chicago, IL) (Tolosa et al., 2015; Bolukbasi et al., 2023)..

2.4. Total RNA isolation and cDNA synthesis

To evaluate of gene expression levels, total RNA extraction from the control and concentration groups was performed using a Qiagen RNeasy Plus Kit (Cat. No/ID: 74034), according to the manufacturer's recommended procedures. Subsequently, the quantity and purity of the RNA were determined using a Nanodrop ND-Spectrometer 1000 device (NanoDrop Technologies, Wilmington, DE, USA) and 1.0 % agarose gel electrophoresis. All isolated RNA samples were treated with DNase enzyme (Thermo Scientific EN0521) to eliminate potential DNA contamination.

The complementary strands of the isolated single-stranded total RNA were synthesized into cDNA using oligo(dT) primer and reverse transcriptase enzyme (RT) with the QuantiTec® Reverse Transcriptase Kit (Qiagen, Germany). After incubation at 42°C for 1 h for cDNA synthesis, the enzyme activity was inhibited by incubation at 70°C for 5 min. An anchored-oligo(dT)18 primer was used due to the long regions of the *CYP1A1* and *BCL*-2 genes.

2.5. The qRT-PCR analyses

The primers for the housekeeping gene β-actin, as well as for the *CYP1A1* and *BCL*-2 genes utilized in this research, were specifically designed based on the genetic data obtained from DLD-1 and CCD18-Co cells available in the gene bank of the National Center for Biotechnology Information (NCBI). Detailed information regarding these genes and sequences of the primers designed specifically for this study are provided in Table 1.

Table 1. The Real-Time PCR primer sequences designed for the genes used in the study.

Genes/ Primers	Length	Gene Bank No	Sequence (5'-3')	Tm (°C)
CYP1A1	2586 bp	BC_023019.1	F:GAACATCCCTATTCTTCGGGG R: GCCATGTGGCCCTTCTGTC	58-60 °C
BCL-2	6492 bp	NM_000633.2	F: CCGTGGGATGCCTTTGACC R: CGGAAACTGAGCAGAGTGGT	58-60 °C
β-Actin	1812 bp	NM_001101.5	F: GGAGGCACCCAGCACTTA R: GCCGATCCACACGGAGTTGA	58-60 °C

Following cDNA synthesis, Real-Time PCR assays were conducted using SYBR Green I Master dye on a Pico Real Time system (Thermo). The mRNA expression levels of *CYP1A1* and *BCL*-2 were assessed by qPCR to investigate the impact of various concentrations of nanomaterials on the expression of cancer-related genes. The PCR conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 25 s, extension at 72°C for 30 s, and melting analysis ranging from 52 to 95°C with a temperature increment of 0.5°C per min. qRT-PCR analysis was performed with three biological replicates, each consisting of three technical replicates, using the optimized conditions obtained.

2.6. Normalization and statistical analysis of qPCR results

The gene expression results obtained as Ct values were normalized using *β-actin* as a housekeeping gene, along with the control conditions utilized in the study (Livak & Schmittgen, 2001). Real-Time PCR reactions for *CYP1A1* and *BCL*-2 genes were concurrently monitored at the most effective concentration determined by MTT analysis, and their peak profiles were recorded. The Ct values of each sample were determined based on the peak profiles. The mRNA levels of the synthesized gene products were quantitatively assessed using the Ct values and Melting Curve Analysis (Kubista et al., 2006). Subsequently, the data were normalized using the $2^{-\Delta\Delta Ct}$ method proposed by Livak and Schmittgen (Livak & Schmittgen, 2001). The mean, standard deviation, standard error and statistical significance of the data were calculated using the statistical software SPSS 25.0 (IBM SPSS, Inc., Chicago, IL).

3. Results

3.1. MTT analysis results

The cytotoxic effects of Cu-flower-shaped nanoparticles obtained from *C. subulata* lichen on DLD-1 and CCD18-Co cell lines were determined by the MTT assay. The cell viability curves (%) were determined based on the doses applied for 24 h, and the half-maximal inhibitory concentration (IC50) values were calculated using Microsoft Excel (Cytotoxicity = test absorbance value / average control absorbance value × 100). The experiments were performed in triplicate, and the results are presented in Table 2.

Table 2. MTT Results of Cu-Flower-Shaped Nanoparticle Applications on DLD-1 and CCD18-Co Cell Lines

DLD-1				CCD18-Co			
Con (µg/ml)	**Absorb**	**% Viability**	**IC50 (µg/ml)**	**Con (µg/ml)**	**Absorb**	**% Viability**	**IC50 (µg/ml)**
C	1,380	100		**C**	1,149	100	
3,91	1,441	110,994		**3,91**	1,193	96,087	
7,81	1,274	98,163		**7,81**	1,068	86,066	
15,63	1,121	86,313		**15,63**	1,046	84,304	
31,25	1,353	104,184	**345,397**	**31,25**	1,143	92,103	**431,649**
62,5	1,295	99,723		**62,5**	1,007	81,098	
125	0,993	76,504		**125**	0,943	75,968	
250	0,712	54,807		**250**	0,788	63,524	
500	0,553	42,602		**500**	0,616	49,631	

Con: Concentration, Absorb: Absorbance, C: Control

Based on these results, the cytotoxic effects of Cu-flower-shaped nanoparticles derived from *C. subulata* lichen on the DLD-1 and CCD18-Co cell lines were evaluated. The IC50 dose of Cu-flower-shaped nanoparticles obtained in DLD-1 cells was 345.397 µg/ml, whereas in CCD18-Co cells, the IC50 dose was 431.649 µg/ml. For this calculation, the formula [Test Substance Absorbance (average) / Control Absorbance (average)] x 100 was used. The cytotoxic effects of Cu-flower-shaped nanoparticles obtained through biological synthesis from *C. subulata* lichen at different concentrations on colon cancer (DLD-1) and healthy colon cells (CCD18-Co) and the corresponding % viability values are shown in Table 2.

3.2. Molecular biological analysis results

The concentration-dependent changes in the expression levels of *CYP1A1* and *BCL-2* genes in DLD-1 and CCD18-Co cells treated with Cu-flower-shaped nanoparticles obtained from *C. subulata* lichen were evaluated separately. The *β-actin* gene was used as a housekeeping gene, and the gene expression results determined as Ct values were normalized accordingly. Real-Time PCR reactions of *CYP1A1* and *BCL-2* genes were monitored simultaneously, and peak profiles were recorded. The Ct values of each sample were determined based on the peak profiles. The mRNA levels of the synthesized gene products were quantitatively analyzed using these obtained Ct values and Melting Curve Analysis. The data were normalized using the $2^{-\Delta\Delta Ct}$ method proposed by Livak and Schmittgen. The mean, standard deviation, standard error, and statistical analysis of the normalized gene expression data were calculated using the SPSS 25.0 Statistical program (IBM SPSS). Based on the results of the normalized gene expression data, the changes in the expression levels of *CYP1A1* and *BCL-2* genes in DLD-1 and CCD18-Co cells treated with Cu-flower-shaped nanoparticles obtained from *C. subulata* lichen, corresponding to each concentration, are provided in Table 3.

Table 3. Mean, standard deviation and standard error of *CYP1A1* and *BCL*-2 gene expression data obtained as a result of normalization of DLD-1 ve CCD18-Co cell lines.

	Mean				Standard Deviation				Standard Error			
	BCL-2		*CYP1A1*		*BCL-2*		*CYP1A1*		*BCL-2*		*CYP1A1*	
Con.(µg/ml)	DLD-1	CCD18-Co	DLD-1	CCD18-Co	DLD-1	CCD18-Co	DLD-1	CCD18-Co	DLD-1	CCD18-Co	DLD-1	CCD18-Co
C	1	1	1	1	-	-	-	-	-	-	-	-
3,91	1,10	1,03	0,82	0,68	0,318	0,573	0,164	0,115	0,184	0,331	0,095	0,067
7,81	1,11	1,64	1,00	0,95	0,448	0,955	0,311	0,459	0,259	0,551	0,179	0,265
15,63	1,19	1,73	1,28	1,59	0,674	1,232	1,060	0,110	0,389	0,711	0,612	0,063
31,25	1,84	2,50	1,56	1,75	0,442	1,050	0,041	0,896	0,255	0,606	0,024	0,517
62,5	3,28	2,81	3,26	1,94	0,792	2,386	0,181	1,012	0,457	1,378	0,104	0,584
125	4,57	3,83	3,93	3,42	2,173	1,513	0,324	1,539	1,254	0,873	0,187	0,889
250	3,13	2,60	2,08	2,70	1,186	0,957	0,409	1,102	0,685	0,553	0,236	0,636
500	1,38	1,41	0,96	1,33	0,107	1,809	0,034	0,008	0,062	1,045	0,020	0,005

Con: Concentration, C: Control

In DLD-1 cells, an approximately 4.57-fold increase in the expression level of the *BCL*-2 gene compared to that in the control group was observed at a dose of 125 ng/ml ($p<0.05$). This increase was 3.28-fold ($p<0.05$) at 62.50 ng/ml and 3.13-fold ($p<0.05$) at 250 ng/ml, which was identified as the highest increase (Table 3). In the expression level of the *CYP1A1* gene, an approximately 3.93-fold increase compared to that in the control group was detected at a dose of 125 ng/ml ($p<0.05$). Changes of 3.26-fold and 2.08-fold were determined at concentrations of 62.50 and 250 ng/ml, respectively. The expression level decreased 0.96-fold at a concentration of 500 ng/ml. This level gradually decreased with increasing concentration above 125 ng/ml. The changes in the expression levels of *CYP1A1* and *BCL*-2 genes in DLD-1 cells treated with Cu-flower-shaped nanoparticles obtained from Cladonia subulata lichen at the determined dose concentrations are shown in Figure 2.

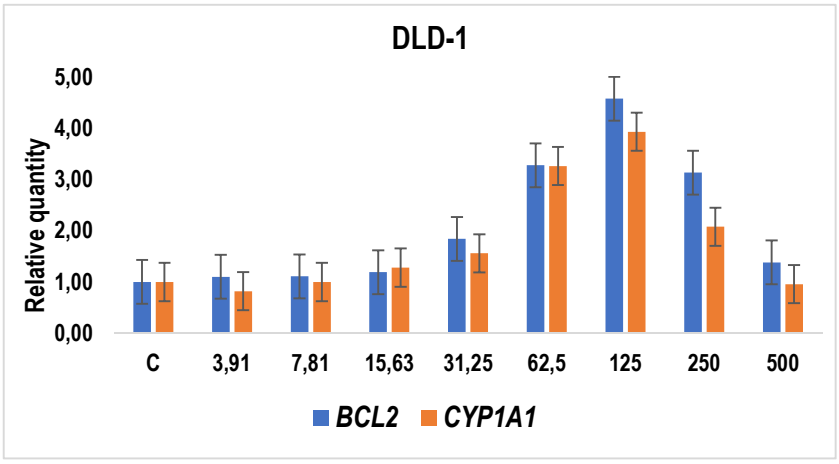

Figure 2. Changes in the expression levels of *CYP1A1* and *BCL*-2 genes in DLD-1 (colon cancer) cell lines treated with Cu-flower-shaped nanoparticles obtained from *C. subulata* lichen at the determined doses.

The results indicated that in DLD-1 cells, the application of Cu-flower-shaped nanoparticles obtained from *C. subulata* lichen led to the highest expression level of the *BCL*-2 gene at a concentration of 125 ng/ml. Thus, it has been demonstrated that compared to the control and other concentrations, *BCL*-2 gene was more expressed at concentration of 62.50, 125, and 250 ng/ml, and exhibited anticancer properties by increasing the expression level of the *BCL*-2 gene, which is effective in the phenomenon of uncontrolled cell proliferation observed in cancer cells. The *CYP1A1* gene, on the other hand, reached the highest expression level with a 3.93-fold increase at the concentration of 125 ng/ml, demonstrating anticancer properties.

In CCD18-Co cells, an increase of approximately 3.83-fold in the expression level of the *BCL*-2 gene was observed at a dose of 125 ng/ml compared to that in the control group. Additionally, an increase of approximately 3.42-fold in the expression level of the *CYP1A1* gene was detected at a dose of 125 ng/ml compared to the control group (Table 3). This increase reached its highest levels at concentrations of 62.50, 125, and 250 ng/ml. The lowest level was 0.68-fold at a concentration of 3.91 ng/ml. The changes in the expression levels of the *CYP1A1* and *BCL*-2 genes in CCD18-Co cells following the application of Cu-flower-shaped nanoparticles obtained from Cladonia subulata lichen at the specified dose concentrations are shown in Figure 3.

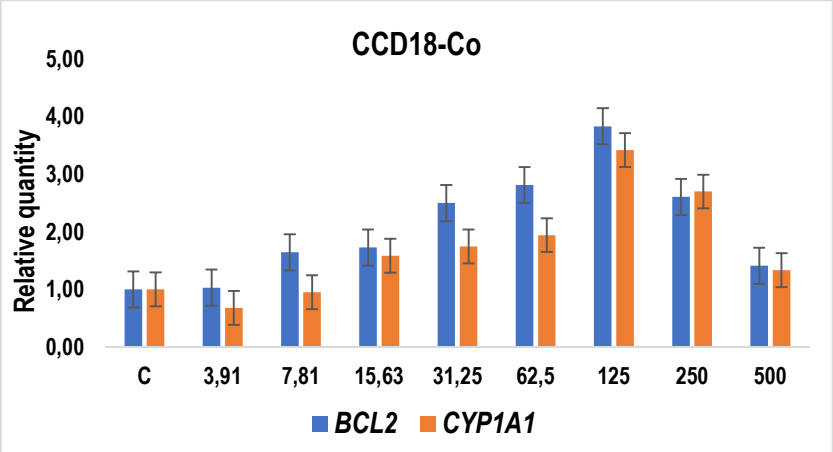

Figure 3. Changes in the expression levels of *CYP1A1* and *BCL*-2 genes in CCD18-Co (healthy normal colon) cell lines treated with Cu-flower-shaped nanoparticles obtained from *C. subulata* lichen at the determined doses.

Upon evaluation of the results, the application of Cu-flower shaped nanoparticles derived from *C. subulata* lichen notably increased the expression levels of the BCL-2 and *CYP1A1* genes in CCD18-Co cells, particularly within the concentration range of 31.25-250 ng/ml, compared to the control group. Consequently, the nanoparticle application exhibited anticancer properties by enhancing the expression levels of the *BCL*-2 and *CYP1A1* genes, which have a protective effect against uncontrolled cell proliferation observed in cancer cells.

4. Discussion

Lichens are a prominent natural source of biological nanoparticles. These symbiotic organisms, formed by the combination of algae and fungi, boast a wide range of biological components and are particularly known for their rich secondary metabolite profiles. Due to these characteristics, they facilitate the synthesis of nanoparticles of various sizes and shapes, especially through interactions with metal salts (Brodo et al., 2001). The species we focused on in our research, from which we biologically synthesized copper-based nanoparticles, is one of 475 different species belonging to the *Cladonia* genus. A previous study, reported that *Cladonia subulata* exhibits cytotoxic activity with the potential for cancer treatment (Hawryl et al., 2020).

Cancer is a disease that leads to an individual's death, negatively impacts the individual, and thereby reduces the quality of life. The most commonly used treatment includes radiotherapy, chemotherapy and surgery. The disadvantages of these treatments are their high cost and the potential for damage to tissues other than cancerous tissue at excessive dosages (Çeşmeli and Avci, 2019). Colon cancer is a type of cancer that begins in the inner walls of the large intestine or the rectum. It typically begins with the development of small, benign tumors called polyps, which can eventually transform into cancer cells. Treatment options vary depending on the cancer stage, its spread, and overall health of the patient (Huang et al., 2017).

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test, introduced by Mosmann in 1983, is a colorimetric assay. Among the biocompatibility tests, the MTT test provides rapid results. It can be used to observe the effects of various compounds or treatment methods on the cells. It is commonly employed to assess the sensitivity of cancer cells and identify potential treatment options. Measuring cellular

responses at the cellular levelcan aid in evaluating the effectiveness of treatment strategies under laboratory conditions (Morgan, 1998; Lin et al., 2019).

In the study conducted based on this information, the cytotoxic properties of flower-shaped copper nanostructures obtained from Cladonia subulata lichen extract were evaluated on DLD-1 and CCD18-Co cell lines using the MTT test. According to the assessment, the IC50 dose of Cu-flower-shaped nanoparticles was found to be 345.397 µg/ml for DLD-1 cells and 431.649 µg/ml for CCD18-Co cells. In literature, Arabinoxylans (AX) and Arabinoxylans-based particles (AXM) were evaluated for their biocompatibility with human colon cells (CCD 841 CoN) using different concentrations of AX and AXM (125, 250, 500, and 1000 µg/ml). The MTT analyses of the human colon cell line CCD 841 CoN in this study showed that neither AX nor AXM inhibited the proliferation of human colon cells (Méndez-Encinas et al., 2019). In the study by Zamani et al. (2018) examined the effect of zinc nanoparticles (ZnNP) on breast cancer ZR-75-1 cell line, reporting a dose-dependent decrease in cell viability with ZnNP application. While 12.5 µM ZnNP was found to be cytotoxic to melanoma cancer cells, it was not cytotoxic to the HaCaT cell line (Zamani et al., 2018).In another study, gold nanoparticles (AuNPs) biosynthesized from Wedelia trilobata plants exhibited dose-dependent toxicity in HCT-15 human colon cells, with efficiency reaching up to an average of 30% (Dey et al., 2018).

In cancer research, gene expression involves processes that determine how cells utilize their genetic material. Gene expression is the process of producing proteins from genesthat regulate the function, growth, proliferation, and differentiation. Specific genes play important roles in cancer development and response to treatment. Therefore, the expression levels of specific genes in cancer cells can be used as biomarkers to predict the course of the disease and the response to treatment (Cartus and Schrenk, 2017). Additionally, cancer treatments can be effective by targeting specific genes or genetic signaling pathways. For instance, suppression of overactive genes or signaling pathways in cancer cells can halt their growth or induce their death. Furthermore, during or after cancer treatment, the expression levels of specific genes in cells can be used to evaluate the treatment response. For example, an increase or decrease in the expression of a particular gene in response to treatment may indicate the treatment effectiveness (Warren et al., 2019). Gene expression analyses provide cancer researchers and clinicians with the opportunity to better understand the biology of the disease and to optimize treatment strategies. Therefore, gene expression studies play a significant role in cancer treatment (Bolukbasi et al., 2023).

In the current study, *Cladonia subulata* (L.) lichen was used for the biological synthesis of copper-nano-flower composite materials. The effects of the synthesized copper-nano-flower composite material on cancer cells (DLD-1 colon cancer cells and CCD18-Co healthy colon cells) were determined by detecting changes in the expression levels of the *CYP1A1* and *BCL*-2 genes, which are involved in the cancer pathway. The identified cells were treated with Cu-based nanostructures at concentrations of 3.91, 7.81, 15.63, 31.25, 62.50, 125, 250, and 500 µg/ml for 24 h. Based on the doses determined through the evaluation of MTT test data, nanoparticle application was performed to detect changes in the expression levels of the *CYP1A1* and *BCL*-2 genes known to be involved in the cancer pathway in DLD-1 and CCD18-Co cell lines.

Molecular analysis indicated that the application of Cu-flower-shaped nanoparticles increased the expression levels of the *BCL*-2 and *CYP1A1* genes. This increase can be associated with an increase in the activity of genes that regulate uncontrolled cell proliferation and apoptosis in cancer cells. In DLD-1 cells, dose-dependent increases in the expression levels of the *BCL*-2 and *CYP1A1* genes were observed following nanoparticle application.The highest expression levels were observed at 125 ng/ml. This increase suggests an increase in the expression levels of *BCL*-2 and *CYP1A1* genes, which are involved in the uncontrolled cell proliferation observed in cancer cells. In CCD18-Co cells, a significant increase in the expression levels of the *BCL*-2 and *CYP1A1* genes was recorded following the application of Cu-flower-shaped nanoparticles. These results indicate that nanoparticle application may also exhibit protective properties against cancer in healthy cells. Therefore, Cu-flower-shaped nanoparticles derived from *C. subulata* lichens are considered potential agents for cancer treatment.

In various studies, the anti-cancer effects of nanoparticles obtained through green biosynthesis have been identified in different cancer cells. Genç et al. performed a green synthesis of gold nanoparticles using a grape (*Vitis vinifera*) seed aqueous extract. Green-synthesized AuNPs exhibited anti-cancer properties in colon cancer cells under in vitro conditions. The results of this study suggest that AuNPs derived from grape seeds could be considered a potential option for colon cancer treatment (Genç et al., 2021). In another study, silver nanoparticles synthesized with rosehip extract were evaluated for their anti-cancer activity in the human colon adenocarcinoma cell line HT29. AgNPs caused a significant

dose-dependent decrease in cell motility. Consequently, the biosynthesized AgNPs demonstrate promising new therapeutic agents for the treatment of human colon cancer (Acar et al., 2019).

This study conducted a series of analyses to evaluate the cytotoxic effects of Cu-flower-shaped nanoparticles obtained from *C. subulata* lichen on colon cancer cells. Initially, the extraction of lichen extracts for the synthesis of Cu-flower-shaped nanoparticles and subsequent preparation of nanoparticles at different concentrations were carried out. Subsequently, the cytotoxic effects of Cu-flower-shaped nanoparticles on DLD-1 (colon cancer) and CCD18-Co (healthy colon) cell lines were determined using a MTT assay. Additionally, the effects of Cu-flower-shaped nanoparticles on gene expression were investigated. For this purpose, the expression levels of *CYP1A1* and *BCL*-2 genes were analyzed using Real-Time PCR. In DLD-1 cells, a significant increase in the expression levels of *BCL*-2 and *CYP1A1* genes was observed following the application of Cu-flower-shaped nanoparticles. This increase is believed to support the anti-cancer properties of Cu-flower-shaped nanoparticles by affecting uncontrolled proliferation of cancer cells. Similarly, in CCD18-Co cells, an increase in the expression levels of *BCL*-2 and *CYP1A1* genes was observed following the application of Cu-flower-shaped nanoparticles. These results indicate that Cu-flower-shaped nanoparticles may also exhibit anti-cancer effects in healthy cells. However, the effects on CCD18-Co cells were less pronounced than those on DLD-1 cells were.

5. Conclusions

In this study, we investigated the cytotoxic effects of Cu-flower-shaped nanoparticles derived from *Cladonia subulata* lichen on colon cancer cells and their effects on gene expression. The results of this study emphasize the potential of nanotechnology in cancer therapy and suggest that nanoparticles obtained from natural sources could be a more environmentally sustainable option. The biological synthesis of Cu-flower-shaped nanoparticles derived from *C. subulata* lichens ensures their biological compatibility and environmental friendliness. These findings indicate that the synthesized Cu-flower-shaped nanoparticles may possess potential anticancer effects on colon cancer cells and healthy colon cells. The obtained data support the evaluation of these nanoparticles as a potential strategy for cancer treatment.This study was conducted only under in vitro conditions, thus it should be noted that further research is needed to draw definitive conclusions regarding the clinical applicability of the obtained findings. Additionally, more studies and clinical research are required to better understand the cellular and molecular mechanisms of nanoparticles and to ascertain the efficacy and reliability of these nanoparticles.

Conflicts of Interests

Authors declare that there is no conflict of interests

Statement contribution of the authors

Conceptualization, E.B., E.A.Y and M.S.A.; methodology, E.B., E.A.Y. and M.S.A.; synthesis of nanoparticles, Ş.P. and M.S.A.; MTT analysis, E.A.Y. and Ş.P.; molecular biological analysis, E.B. and Ş.P.; writing-original draft preparation, E.B., E.A.Y. and M.S.A.; writing-review and editing, E.B. and M.S.A. The authors carefully reviewed the final version of the manuscript and agreed that it accurately represented the findings and conclusions of the study. Also carefully was reviewed the final draft to ensure that it met high standards for quality and accuracy. The authors read and agreed to the final version of the manuscript.

Statement of ethics

There is no need for an ethics committee decision for the studies in the article.

Acknowledgements:

The authors gratefully acknowledge the financial support of this work by Amasya University Scientific Research Unit (Project No: FMB-BAP 23-0594). And also, the authors acknowledge to Prof. Dr. Mehmet Gökhan HALICI at Erciyes University, Science Faculty and Department of Biology and Assoc. Prof. Dr. Fatih Dogan KOCA at Erciyes University, Faculty of Veterinary, Department of Aquatic Animal and Diseases, for their valuable support.

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