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Apoptosis stimulating effect of *Cladonia furcata* on breast cancer cells via PI3K/Akt/mTOR pathway

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Lichens are common and resistant organisms consisting of fungi and algae or cyanobacteria. Their components have shown antibacterial, antimutagenic, antiviral, antifungal, and anticancer properties. Therefore, this study aimed to determine the protective effects of *Cladonia furcata* on MDA-MB-231 breast cancer cells. The XTT assay, comet assay, and antioxidant assay were used. The protective effect of *C. furcata* on MDA-MB-231 cells was determined in control groups and treatment groups (10-200 µg/mL) with an LD_{50} value of 60 µg/mL. When treatment groups, *C. furcata* applied to MDA-MB-231 cells at increasing doses were compared with the control group, it was determined that there was a decrease in enzyme activity and an increase in the amount of MDA. DNA damage was revealed by the comet test. It was determined that the cells used PI3K/Akt/mTORmetabolic pathways while entering apoptosis. The anticancer effects of *C. furcata* were determined according to the dose-dependent deletion and expression on disappearing the band formation obtained from cell DNA. In our study, *C. furcata* extract showed beneficial effects on breast cancer cells by triggering apoptotic mechanisms at certain doses and led to the elimination of cells.

Keywords: MDA-MB-231, *Cladonia furcata,* XTT assay, Antioxidant test, TUNEL assay, Immunocytochemistry test, RAPD-PCR

Lichens are mutualistic symbiotic organisms formed through the symbiotic association of algae and fungi, based on morphological and physiological characteristics, exhibiting resilient structures[1]. Since the defensive nature of lichens has anticancer potential against a wide range of cancers, their pharmaceutical utilization has begun to proliferate[2]. Lichens have been utilized for medical purposes since ancient times[3]. Chemical and pharmacological investigations have substantiated the capacity of lichen secondary metabolites to exhibit biological activity, thereby establishing their potential use as antibiotics, UV absorption, and antioxidants[4]. The possession of antioxidant activity by lichens has suggested that their ingestion as a dietary source may enhance lifespan and antimutagenic activity[5]. *Cladonia furcata* lichen can also be found in shady meadows, valley floors, roadsides, rocky outcrops and forests[6].

MDA-MB-231 represents one of the frequently employed human breast cancer cell lines in research laboratories. These cells possess a triple-negative, aggressive, and invasive phenotype. The chemosensitivity of these cells is classified as moderate[7,8]. The MDA-MB-231 cell line exhibits an adhesive trait, facilitating its convenient utilization in *in vitro* culture studies within the field of biology[9,10]. The findings derived from a prior study have provided information regarding the fumarprotocetraric acid content within the *C. furcata* extract. The amount of fumarprotocetraric acid in *C. furcata* extract was found to be 3.48 mg[11]. These results make it clear that we need to do more research to fully understand the pharmacological potential of the *C. furcata* extract. This is due to the connection between fumarprotocetraric acid and biological and antioxidant activities.

The XTT assay employs quantitative methods to assess cytotoxicity in cells. Dehydrogenase enzymes in living cells reduce the XTT reagent in this method, giving formazan crystals a purple colour. As the live cell density increases, the number of formazan crystals also increases, and the viability of cells is determined by measuring these crystals spectrophotometrically[12].

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The comet assay is one of the common methods used to assess DNA damage in living organisms[13,14]. The presence of genetic damage or deficiency in appropriate repair mechanisms is a well-known factor in the etiology of cancer and other non-infectious diseases[15].

Non-enzymatic pathways form MDA (malondialdehyde), a toxic by product of lipid peroxidation. Measurement of MDA levels is used to assess levels of lipid peroxidation[16]. Antioxidants are substances that reduce oxidative stress and thus prevent cellular damage. Long-term oxidative stress can lead to structural changes in DNA, carbohydrates, lipids, and proteins[17-19]. Intracellular antioxidant defense systems: include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SOD is an enzyme responsible for the elimination of superoxide radicals, a combination of oxygen and hydrogen peroxide. CAT is an enzyme that reduces or breaks down hydrogen peroxide (H_2O_2)[20,21]. GPx is an enzyme that helps protect cells against the harmful effects of oxidative damage. In cells, GPx can metabolize organic hydroperoxides. This enzymatic activity helps prevent cell damage and maintain cellular function[18]. Free radicals readily bind to the unsaturated bonds of cholesterol and fatty acids in the cell membrane, triggering a phenomenon known as lipid peroxidation[22,23].

Apoptosis, also known as programmed cell death, is defined as a form of cell death in complex organisms where cells that are no longer needed for maintaining homeostasis or have impaired functions undergo a predetermined series of molecular events leading to death without causing damage[24]. Necrosis is a random and uncontrolled process of cell death[25]. Cell culture experiments, where cells grow and maintain themselves in a controlled environment, can also evaluate the degree of apoptosis using the TUNEL assay[26].

Immunocytochemistry uses specific antibodies to label target proteins inside the cell. Immunocytochemistry allows us to gain insights into the function and organization of cells by combining morphological information with protein expression[27-29].

RAPD (random-amplified polymorphic DNA) PCR is one of the molecular biology techniques used to detect genetic diversity and phylogeny. Through random fragmentation and amplification of DNA fragments it enables unique polymorphic patterns to be obtained in different organisms and offers the possibility of detecting novel gene mutations[30,31]. This study investigated the potential anticancer effects of C. furcata on the MDA-MB-231 breast cancer cell line by subjecting them to increasing doses. We investigated these effects using the TUNEL method, XTT assay, comet assay, RAPD method, and immunocytochemistry method. Additionally, we assessed the levels of malondialdehyde (MDA) and antioxidant enzyme activities.

Materials and Methods

Lichen material and reagents

Cladonia furcata (Huds.) Baumg was collected from the Arsuz district of Hatay province, Turkey, in 2021. The collected lichen specimens were identified using identification keys according to their morphological and anatomical characteristics. The Bogazliyan Vocational School Lichen Laboratory houses the collected lichen samples.

Preparation of the extracts

Air-dried *C. furcata* (10 g) was pulverized. The powdered lichens were extracted using 70% methanol (MeOH) according to the method of Kocakaya *et al.*[11]. The extracts were filtered and removed from the solvent under vacuum (37°C). The resulting extracts were lyophilized and powdered. It was then stored at 20°C for use during analysis[32].

Cell culture

In this study, while analyzing the protective effects of *C. furcata* on breast cancer, MDA-MB-231 cells were preferred as the breast cancer cell line. The MDA-MB-231 (estrogen receptor-negative) breast cancer cell line was procured from the American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured in RPMI-1640 (Roswell Park Memorial Institute 1640 Medium). RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotic, and vitamin B12 and PABA were used. The cell line used was cultured at 37°C in a 5% CO_2 atmosphere.

XTT assay

96-well plates were used for cell seeding. An equal number of cells (10^4) were seeded into each well and placed in the incubator. After 24 h, various doses of *C. furcata* were applied to the cells. Treatment doses were 10 µg/mL, 30 µg/mL, 60 µg/mL, 120 µg/mL, and 200 µg/mL. These doses were applied through dilution with a culture medium and

investigated alongside a control group. For the evaluation of substance effects, cell plates were placed in the incubator and incubated for 48 h. The XTT assay (Cell Proliferation Kit, Roche) mixture (50 µL) was then added to each well and left to sit for 18 h. After an 18 h incubation in the wells, the formed formazans, which were present at different densities, were measured at 570 nm absorbance using a microplate reader spectrophotometer.

Comet assay

The MDA-MB-231 cells were detached from the flask surface using trypsin, and cell counting was performed using a cell counter. Subsequently, the cells were seeded into 6-well plates and incubated overnight in the incubator. Then, lichen application groups were put into the wells at 10 µg/mL, 30 µg/mL, 60 µg/mL, 120 µg/mL, and 200 µg/mL concentrations. The plates were then left to grow for two more nights. Samples were collected from each lichen application group and the control group, pipetted onto slides with a 1:1 ratio of agarose (LMA), and covered with coverslips. The slides were then allowed to solidify at +4°C for 20 min. The cells in the solidified agarose were subjected to lysis, pre-electrophoresis, electrophoresis, and dH_2O treatments and then air-dried for 15 min. Following this stage, the samples were stained with ethidium bromide (EtBr) and examined under a fluorescence microscope to capture images.

Determination of malondialdehyde level and SOD, CAT, and GPx determination of enzyme activities

The 'Bioassay Technology Laboratory ELISA Kit' was utilized to determine enzyme activities. The cells were seeded in a flask and incubated for 24 h. Subsequently, the lichen application groups were introduced. After a two-night incubation, the cells were detached from the flask surface using trypsin and then centrifuged at 1500 RPM for 15 min. Following this step, the experimental protocol of the kit was implemented, and the measurements were taken at 450 nm absorbance using a spectrophotometer.

Examination of apoptotic cells during cell death by the TUNEL method

In this research, 8-well slides were used. Each well was seeded with 10^5 cells, and the cells were incubated at 37°C in a 5% CO_2 environment for 24 h to facilitate cell adhesion. The cells were then prepared by applying doses of 10 µg/mL, 30 µg/mL, 60 µg/mL, 120 µg/mL, 200 µg/mL, and a control group, resulting in six experimental groups. The TUNEL method was utilized, using the Thermo Fisher TUNEL assay kit and protocol. DAPI (Invitrogen) was used as the nuclear counterstain.

Investigation of the PI3K/Akt/mTORpathway

In this study, 8-well slides were utilized. Each well was seeded with 10^5 cells, and the cells were incubated at 37°C in a 5% CO_2 environment for 24 h to facilitate cell adhesion. Application doses of 10 µg/mL, 30 µg/mL, 60 µg/mL, 120 µg/mL, 200 µg/mL, and a control group were administered to the wells, resulting in six experimental groups. After 24 h, the wells were washed with PBS buffer and fixed with paraformaldehyde. Subsequently, they were washed three times with PBS buffer. Triton-X incubation was performed on ice, and the wells were washed with PBS buffer again. H_2O_2 was added, followed by another wash with PBS. A large volume of ultra V block was added, and incubation was performed for 1 h. Then, individual antibodies were added to separate wells and left to incubate overnight. The biotinylated anti-polyvalent solution was added and incubated for 30 min. Streptavidin peroxidase was added to the wells and incubated for 30 min. DAB was applied to the cells, and finally, the wells were washed three times with dH_2O. A mounting medium was added, and the slides were covered using a long lamella. The images were captured under a light microscope.

Implementation of RAPD-PCR using DNA samples isolated from cells

In this study, a 10 bp OPB 07 (5'-GGT GAC GCA G-3') DNA primer was used to obtain DNA samples from MDA-MB-231 cells. The necessary preparations for RAPD-PCR were performed on an ice-cold surface. The samples were loaded into the thermal cycler for RAPD-PCR procedures. The RAPD-PCR conditions were set at an initial temperature of 94°C and a total of 150 min and 40 cycles. 1.5% agarose gel was prepared for the running of PCR samples. The samples, including the marker, were loaded into the electrophoresis buffer and mixed with dye. The electrophoresis process lasted 35 min at 250 volts. The gels, with the addition of ethidium bromide (4 µg/mL), were soaked in pure water for 25 min. A gel imaging system was used to visualize the DNA bands amplified with primers.

Statistical analysis

Data were analysed with one-way analysis of variance (ANOVA) and the Tukey test using Windows SPSS 11.5 computer software and $P<0.05$ value was considered statistically significant.

Results

XTT assay

Spectrophotometric measurements determined that *C. furcata* reduced the proliferation rate of MDA-MB-231 cells and had a protective effect against cancer in experiments where all cells on the microplate, including the control group with only the growth medium, were healthy and densely confluent. Fig. 1 presents the results. The cell viability was determined to be halved compared to the control group, with an LD_{50} value of 60 µg/mL. Blank values were considered in the calculations, and the results were obtained by subtracting these values.

Determination of SOD, CAT, and GPx enzyme activities and measurement of malondialdehyde level

When the groups applied with increasing doses of *C. furcata* were compared to the control group in terms of SOD, CAT,and GPx enzyme activities, it was observed that oxidative stress increased in a dose-dependent manner, leading to a reduction in enzyme activities. The decrease in enzyme activities depicted in the column graphs reflected the extent of oxidative damage. Furthermore, a significant increase in MDA levels was noted in MDA-MB-231 cells when compared to the groups treated with increasing doses of *C. furcata*. This elevation signified an upsurge in lipid peroxidation intermediates and consequent damage to the cell membrane. Fig. 2 presents the results.

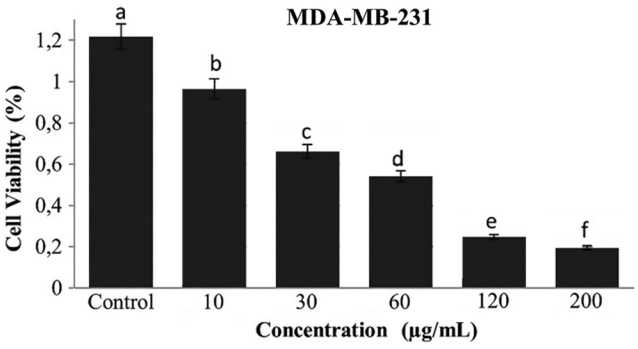

Fig. 1 — The effects of *C. furcata* on MDA-MB-231 cell viability (groups with different letters on the columns indicate significant differences ($P<0.05$) between them).

Examination of DNA damage under alkaline conditions using the comet assay

The genotoxic measurements of *C. furcata* applied to the MDA-MB-231 cancer cell line at different concentrations were determined using the comet assay, which allowed us to measure DNA damage. Fig. 3 visually presents the damage-dependent tail lengths. The control in the figure indicated no DNA damage and intact nuclei, while the increase in tail length with increasing doses revealed the damage to DNA strands as they moved away from the nucleus. The average values of DNA damage parameters such as DNA%, tail length, and tail moment observed in the cells were statistically calculated and presented in Table 1.

Examination of the PI3K/Akt/mTORpathway in cell death by immunocytochemistry method

The immunocytochemistry test images, using DAB staining, to determine which pathways cancer

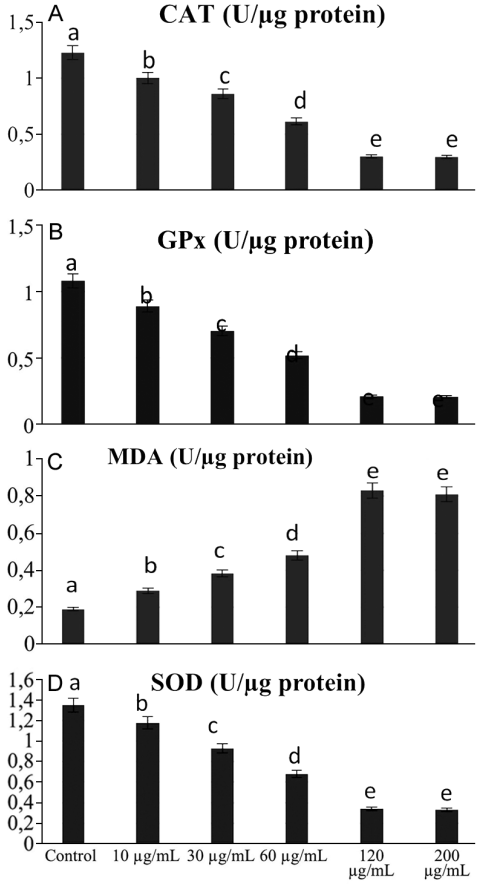

Fig. 2 — Depicts the comparison of SOD, CAT, and GPx enzymes activities and MDA levels between the control group and the increasing application groups of *C. furcata*. Groups with different letters on the columns indicate statistically significant differences between them ($P<0.05$).

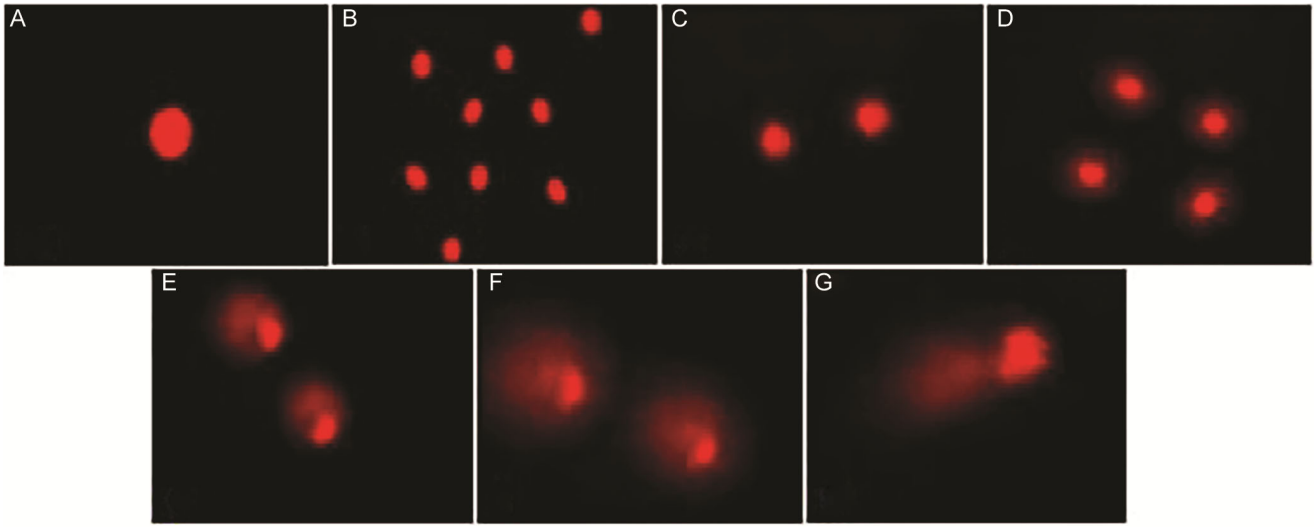

Fig. 3 — Photographs of MDA-MB-231 cells in lichen application groups captured using a fluorescence microscope. (A, B) Control group; (C) 10 µg/mL lichen- applied group; (D) 30 µg/mL lichen- applied group; (E) 60 µg/mL lichen-applied group; (F) 120 µg/mL lichen- applied group; (G) 200 µg/mL lichen- applied group.

Table 1 —Mean values±SD of DNA damage in control and treatment groups of MDA-MB-231 cells. Significance at P<0.05. Within each column, means superscript with different letters are significantly different.

Groups	Tail DNA % ±SD	Tail Length ±SD	Tail Moment ±SD
Control	9.2±1.9	1.6±0.02	0.14±0.0003
10 µg/mL	15.7±3.4[a]	9.02±1.15[a]	1.41±0.39[a]
30 µg/mL	28.3±5.7[b]	12.44±2.12[b]	3.52±0.12[b]
60 µg/mL	41.11±8.3[c]	18.42±1.32[c]	7.57±0.10[c]
120 µg/mL	86.7±15.2[d]	21.01±2.23[d]	18.21±0.33[d]
200 µg/mL	98.4±21[e]	24.78±3.11[e]	24.38±0.65[e]

cells utilize in the apoptotic process, are presented in Fig. 4. When using PI3K/Akt/mTOR antibodies separately, the brown spots indicating a dose-dependent regulation of the antibodies demonstrated that apoptosis occurred through these three pathways, as evidenced by the images compared to the control.

Investigation of apoptotic cells in cell death by the TUNEL method

It can detect apoptotic cells and DNA fragmentation in cells using a fluorescent dye called DAPI and shows apoptotic cells in Fig. 5. The brightly stained cells represent cells undergoing apoptosis, while the non-emitting cells represent those that did not undergo apoptosis. Images taken under two different wavelengths, green and blue, using the Zeiss vert.A1 inverted microscope, were presented.

Analysis of cell polymorphisms using the RAPD-PCR method

DNA genotoxicity and polymorphisms of MDA-MB-231 cells, amplified through PCR with the

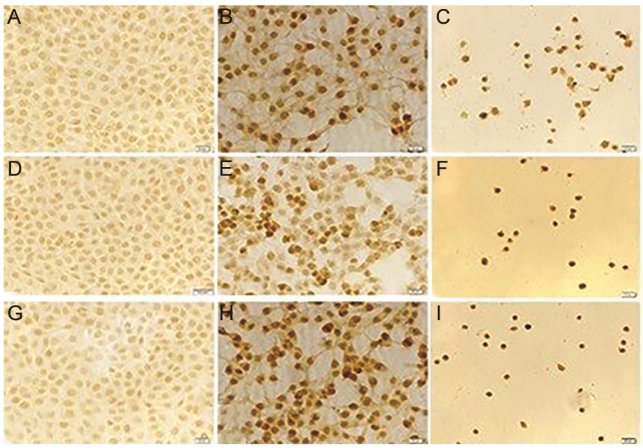

Fig. 4 — Immunocytochemical staining and light microscope images of PI3K/Akt/mTOR proteins applied to MDA-MB-231 cells and their activation status. (A) PI3K control group; (B) PI3K with 60 µg/mL *C. furcata* application; (C) PI3K with 120 µg/mL *C. furcata* application. (D) AKT control group; (E) AKT with 60 µg/mL *C. furcata* application; (F) AKT with 120 µg/mL *C. furcata* application. (G) mTOR control group; (H) mTOR with 60 µg/mL *C. furcata* application; (I) mTOR with 120 µg/mL *C. furcata* application.

appropriate primer (OPB 07 DNA primer), were visualized on an agarose gel under UV light, and the band images are presented in Fig. 6. A marker (M) was used to compare the changes in DNA. While 8 bands were observed in the control group, the group treated with 60 µg/mL of *C. furcata* showed 6 bands. In the group applied with 120 µg/mL of *C. furcata*, the bands became faint, and the number of bands decreased to 5. The results show that the application of *C. furcata* to breast cancer cells

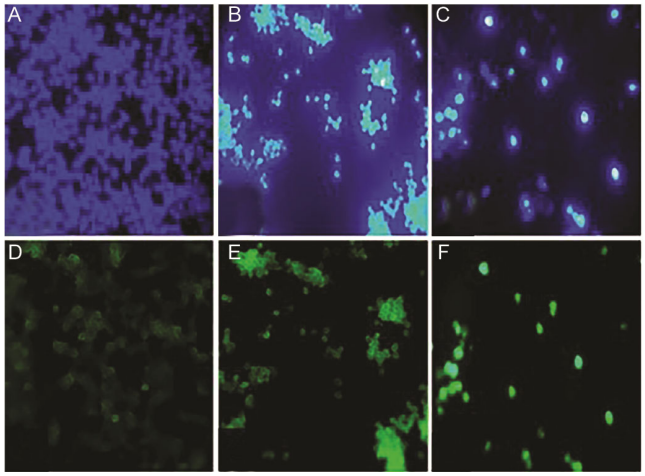

Fig. 5 — Shows the images of apoptotic cells in MDA-MB-231 cancer cells applied with *C. furcata*, detected by DNA fragmentation using DAPI staining at different wavelengths, and the fluorescence microscope images of protein activation status. Images were captured using two different wavelengths, blue and green, on a Ziess vert.A1 inverted microscope. (A) Blue light control group; (D) Green light control group; (B) Blue light 60 µg/mL *C. furcata* applied group; (E) Green light 60 µg/mL *C. furcata* applied group; (C) Blue light 120 µg/mL *C. furcata* applied group; (F) Green light 120 µg/mL *C. furcata* applied group.

resulted in deletions and reduced expression compared to the control group, as depicted in the given Fig. 6.

Discussion

Women who have breast cancer risk factors are considered to be in high-risk groups. Research shows that avoiding obesity, regular exercise, following a healthy diet, avoiding alcohol consumption and long-term hormone replacement therapy can significantly reduce the risk of breast cancer[33]. Many countries have long used lichens to treat human diseases. Studies have revealed that these slow-growing organisms, which are known to produce a wide range of secondary metabolites, possess various life activities[34]. Lichens are commonly found in multiple terrestrial ecosystems, and studies investigating different groups of lichens have demonstrated significant progress and success in their potential anticancer abilities[35]. Lichens have been characterized as a source of biologically active enzymes, polysaccharides, and fatty acids that may possess pharmacological potential[36,37]. Lichens are found in most terrestrial ecosystems around the world, and lichen substances have achieved significant evolutionary success with a broad spectrum of anticancer activity[38].

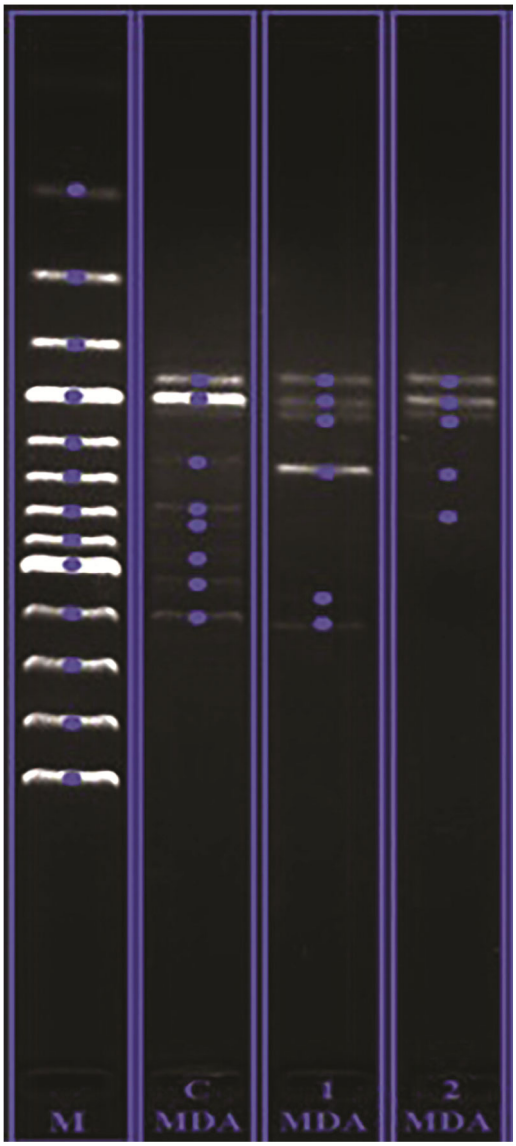

Fig. 6 — Gel images of RAPD amplification products run on electrophoresis. M (Marker); It is essential for ensuring the accuracy and reliability of the amplification. MDA-MB-231 cells control group C-MDA; MDA-MB-231 cells applied with 60 µg/mL of *C. furcata* 1-MDA; MDA-MB-231 cells applied with 120 µg/mL of *C. furcata* 2-MDA.

Studies have revealed various biological effects of *Cladonia* species. In terms of antioxidant potential, they exhibited a mechanism to reduce oxidative stress in cells and prevent the harmful effects of free radicals. Atranorin and oxalic, propionic, tartaric, butyric, malonic, lactic, citric, maleic, fumaric, styric, succinic, norstitic, usnic, lecanoric, orsellinic and furmarprotocetraric acids are found in various *Cladonia* species and have anticancer effects[39]. Furthermore, regarding their antiproliferative potential, *Cladonia* extracts have been found to

inhibit the proliferation and growth capabilities of cancer cells, thereby preventing cancer development[40].

In one study lichen extracts and isolated lichen compounds were reported to interact with all biological entities currently identified as responsible for tumour development. Furthermore, lichen-based compounds induce the killing of cells through the processes of apoptosis, autophagy and necrosis, which positively modulate against uncontrolled division of cells. Many lichen-based compounds, alone or in combination with other anticancer compounds, have been shown to have potential anticancer activity against a wide range of cancer cells[41]. Atranorin and fumarprotocetraric acid are significant metabolic acids present in the extract of *C. furcata*[36]. Lichens' pharmaceutical effects arising from their defense mechanisms are associated with their highly important anticancer potential. The anticancer effect of these organisms, which possess multiple metabolites, has been effectively observed in various *in vitro* cancer models[2,42]. This study measured the absorbance values of formazan crystals formed by tetrazolium salts using XTT tests. XTT allows us to evaluate the substance's cell viability, cell proliferation, and cytotoxicity without the need for an additional solubilizer, and it provides information about the highest viability group at higher concentrations. The colour intensity resulting from formazan crystals is directly related to the number of viable cells, while non-viable cells do not show colour development due to the lack of NADH reduction[12,43]. In this study, the *C. furcata* lichen was applied to the human breast cancer cell line MDA-MB-231, and its cytotoxic effect on cancer cells was assessed using the XTT proliferation assay. In the application groups of *C. furcata* at concentrations of 10 µg/mL and 30 µg/mL, a decrease in cell viability was observed. The LD_{50} value, which represents the concentration at which 50% of the cells died, was determined to be 60 µg/mL. The LD_{100} value, which resulted in complete cell death, was found to be 120 µg/mL.

The comet assay measures genotoxic effects. This test detects single and double-strand DNA breaks, alkali-labile sites, and sites of faulty repair, thereby enabling the detection of DNA damage and providing the opportunity to observe DNA damage. It detects primary DNA damage that could lead to mutations[44,45]. In our study, it was observed that increasing doses of *C. furcata* treatment on MDA-MB-231 cells induced DNA damage in malignant cells and demonstrated a protective effect against cancer through the application of lichens. Compared to the control group, the most significant increase in DNA length and decrease in cell count were observed, indicating the dominant effect on cancer cells at a dose of 60 µg/mL.

One study evaluated the neuroprotective role of methanol extracts of *Dactylina arctica*, *Nephromopsis stracheyi*, *Tuckermannopsis americana,* and *Vulpicida pinastri* in a hydrogen peroxide (H_2O_2) oxidative stress model in the neuroblastoma cell line "SH-SY5Y cells". The results of the study showed that among the four methanol lichen extracts tested, *N. Stracheyi* exerted its neuroprotective activity by inhibiting ROS and increasing CAT activity, while *V. pinastri*, *D. arctica,* and *T. Americana* prevented ROS overproduction and preserved enzyme activity[46]. Superoxide dismutase (SOD) is an enzymatic antioxidant that catalyzes the dismutation of superoxide radicals into O_2 and H_2O_2[47,48]. In this study, the results of SOD activity were evaluated comparatively between the *C. furcata* applied groups and the control group. A decrease in affected SOD activity was observed in the examined groups with different doses of *C. furcata*. The toxic effects of H_2O_2, a reactive oxygen species, on cells were neutralized and rendered ineffective by CAT and GPx. The CAT enzyme exhibited its activity by catalyzing the breakdown of H_2O_2 into water and oxygen[49,50]. In this study, the results of SOD activity were comparatively evaluated between the *C. furcata* treatment groups and the control group. The groups treated with different doses of *C. furcata* showed a decrease in SOD activity. CAT and GPx enzymes neutralized and rendered ineffective reactive oxygen species, such as H_2O_2, which exerted toxic effects on cells. The CAT enzyme catalyzed the decomposition of H_2O_2 into water and oxygen. According to the comparison results, a significant decrease in CAT enzyme activity was detected between the group treated with 60 µg/mL of lichen and the control group, indicating a potential increase in enzymes capable of catalyzing H_2O_2 in the body and an inclination towards H_2O_2 accumulation. GPx, an enzymatic antioxidant, protects the cell from the hydroxyl radical activity of H_2O_2 by utilizing GSH, leading to the formation of glutathione disulfide (GSSG) and H_2O. In conditions with high H_2O_2 levels inside the cell, CAT played a vital role, whereas GPx

exhibited higher efficiency at lower concentrations. Among the treatment groups, a decrease in GPx activity was observed compared to the control groups. This suggests a high accumulation of H_2O_2 within the cells in these circumstances[18]. MDA, by chemically intervening in ion transport activities and disrupting the integrity of the cell membrane, triggered intracellular cascade reactions[51]. In our study, it was determined that *C. furcata* reduced the activities of SOD, CAT, and GPx enzymes and significantly increased MDA levels in MDA-MB-231 cells. When the results of antioxidant enzymes and MDA were evaluated together, it was found that *C. furcata* increased oxidative stress in MDA-MB-231 cells.

When evaluating the TUNEL test results, we observed that cancer cells were directed to apoptosis at appropriate doses, indicating a potential anticancer effect. This immunocytochemistry study clarified the molecular mechanism of apoptosis, identifying the PI3K/Akt/mTOR pathway as the utilized cell death signalling pathway. A correlation was also found between the TUNEL test and the immunocytochemistry results obtained from the application groups of this lichen. Our results suggest that *C. furcata* induces DNA compaction and DNA fragmentation in cells, leading to an antiproliferative effect in cancer cells. Further in-depth investigations in the future could potentially facilitate its targeted control. The PI3K/Akt/mTOR signalling pathway is a key therapeutic target in breast cancer, and the fact that it controls cell death signals is very important for treating breast cancer. Research has shown that disruptions in mTOR signalling contribute to various diseases, including cancer[52]. Intracellular oxidative stress, DNA damage, and polymorphisms exert an opposing effect on cancer cell proliferation by inducing cell death through the utilization of the PI3K/Akt/mTOR signalling pathway. Based on the obtained immunosignal results, the PI3K/Akt/mTOR signalling pathway, which is a crucial step in autophagy initiation, showed a positive outcome in DAB staining, indicating an upregulation in protein regulation. Increasing doses of *C. furcata* induced apoptosis through the PI3K/Akt/mTOR cellular signalling pathway, directing the cells toward autophagy. The regulatory capability of *C. furcata* on the mTOR signalling pathway in cancer cells is anticipated to contribute to its cancer-preventive traits.

Due to the polymorphic characteristics exhibited by primers, genomic alterations in human breast cancer can be identified[53]. RAPD markers are employed to reveal differences among genotypes. Random primers enable the exploration of genetic variations in different regions of the genome, albeit randomly determining the entire genome[54]. In the RAPD-PCR test, using universal primers, polymorphic DNA bands and various polymorphisms and mutations were observed. To compare the bands and polymorphisms induced by *C. furcata* applied groups with the control in MDA-MB-231 breast cancer cells, they were run together on an agarose gel to reveal the differences resulting from damage to the bands. Fragments in the genomic DNA of MDA-MB-231, where *C. furcata* lichen induced DNA damage, were identified along with band deformations.

Conclusions

The research conducted in this study accentuates the potential anticancer attributes of *Cladonia furcata* lichen and its bioactive constituents. The XTT proliferation assay substantiates the noteworthy cytotoxic effects of C. *furcata* on human breast cancer cells, revealing a dose-dependent decline in cellular viability. The determination of an LD_{50} value of 60 µg/mL underscores its effectiveness in impeding the proliferation of cancer cells. Furthermore, the study revealed that *C. furcata* induces DNA damage in malignant cells, as demonstrated by the comet assay. This genotoxic effect, in conjunction with the observed increase in DNA length and decrease in cell count, indicates its potential as a protective agent against cancer. The research also elucidated the antioxidant properties of *C. furcata* and its potential to mitigate oxidative stress in cancer cells. It was observed that the activities of SOD, CAT, and GPx enzymes were influenced by *C. Furcata* treatment, resulting in an increase in oxidative stress, as indicated by elevated MDA levels. Furthermore, the study delved into the mechanisms of cell death induced by *C. furcata,* unveiling its capacity to initiate apoptosis in cancer cells. The PI3K/Akt/mTOR pathway was identified as a central mediator in this process, contributing to the regulation of cell death signals. RAPD-PCR was used to look closely at DNA polymorphisms in cancer cells. The results showed that *C. furcata* caused genetic changes, which supports its potential as a cancer treatment. Cytotoxic effects in MDA-MB-231 human breast

cancer cells, elucidation of antioxidant properties, and the anticancer role of the lichen extract applied by determining the effective values of SOD, CAT, GPx, and MDA of oxidative stress pathways were determined. PI3K/Akt/mTOR pathway, TUNEL, and RAPD results also show the complicated cell interactions that cause *C. furcata* to fight cancer in the study of molecular mechanisms.

Author contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mustafa KOCAKAYA, Elif AKBULUT and Zekiye KOCAKAYA. The first draft of the manuscript was written by Mustafa KOCAKAYA, Elif AKBULUT and Zekiye KOCAKAYA all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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