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Broccoli (Brassica oleracea var. italica) extract's potential therapeutic targets on human breast cancer (MDA-MB-231) using the AgNOR detection method

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Research Article

History

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ABSTRACT

Background: In this study, the in vitro effect of the extract obtained from mature broccoli on the breast cancer cell line was aimed to be determined.MDA-MB-231 cells were exposed to broccoli extract at 37°C and 5% CO2 for different durations (24 and 48 hours) and doses (125 and 250 µl/ml). At the end of the incubation period, the viability, apoptosis, cell cycle, and AgNOR protein status of MDA-MB-231 cells were examined using the Muse Cell Analyzer.In the groups treated with broccoli extract, a decrease in the percentage of viable cells and a significant increase in the percentage of early and total apoptosis were observed for both doses compared to the control group. In the cell cycle analysis, there was an increase in the number of cells in the S phase in all the groups. It was observed that the groups treated with broccoli extract caused a delay in the cell cycle progression at the transition to the S checkpoint. AgNOR staining results further supported the cell cycle and apoptosis findings. The AgNOR number and TAA/NA ratio decreased in the 125 µl/ml broccoli extract group after 24-48 hours and were found to be statistically significant compared to the control group. It was determined that broccoli extract induced apoptosis in breast cancer cells through various mechanisms and inhibited cell viability and growth. These results were consistent with the findings related to AgNOR protein synthesis.
The study demonstrated that the consistent and proper consumption of broccoli may be effective in preventing cancer formation and slowing its progression.

Keywords: Diabetic nephropathy, microalbuminuria, serum uric acid/creatine ratio

Brokoli (Brassica oleracea var. italica) Ekstraktının İnsan Meme Kanseri Hücrelerine (MDA-MB-231) Etkisi ve AgNOR Tespit Yöntemi ile Analizi

Süreç

Geliş: 07/01/2023
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ÖZ

Arka Plan: Bu çalışmada, olgun brokolinin ekstraktının meme kanseri hücre hattı üzerindeki in vitro etkisinin belirlenmesi amaçlanmıştır.MDA-MB-231 hücreleri, 37°C ve %5 CO2'de farklı süreler (24 ve 48 saat) ve dozlar (125 ve 250 µl/ml) için brokoli ekstraktına maruz bırakıldı. İnkübasyon süresinin sonunda, MDA-MB-231 hücrelerinin canlılık, apoptozis, hücre döngüsü ve AgNOR protein durumu Muse Hücre Analizörü kullanılarak incelendi.
Brokoli ekstraktı ile tedavi edilen gruplarda, canlı hücre yüzdesinde bir azalma ve erken ve toplam apoptozis yüzdesinde kontrol grubuna göre anlamlı bir artış gözlendi. Hücre döngüsü analizinde, tüm gruplarda S fazındaki hücre sayısında bir artış gözlendi. Brokoli ekstraktı ile tedavi edilen grupların, S kontrol noktasına geçişte hücre döngüsü ilerlemesinde bir gecikmeye neden olduğu gözlendi. AgNOR boyama sonuçları, hücre döngüsü ve apoptozis bulgularını daha da destekledi. 24-48 saat sonra 125 µl/ml brokoli ekstraktı grubunda AgNOR sayısı ve TAA/NA oranında azalma gözlendi ve kontrol grubuna göre istatistiksel olarak anlamlı bulundu. Brokoli ekstraktının, meme kanseri hücrelerinde çeşitli mekanizmalar aracılığıyla apoptoza neden olduğu ve hücre canlılığını ve büyümesini inhibe ettiği belirlendi. Bu sonuçlar, AgNOR protein sentezi ile ilgili bulgularla tutarlıydı. Çalışma, brokolinin tutarlı ve uygun tüketiminin, kanser oluşumunu önlemede ve ilerlemesini yavaşlatmada etkili olabileceğini göstermiştir.

Anahtar sözcükler: Diyabetik nefropati, mikroalbünüri, serum ürik asit /creatin oranı

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Introduction

Cancer is a highly lethal disease characterized by uncontrolled cell proliferation and invasive metastasis. It stands out as a leading cause of death and a significant public health issue in many countries, driven by rapid population growth and an aging population worldwide. Breast cancer is the most prevalent cancer type among women in Turkey and worldwide.1 Chemotherapy is a widely employed cancer treatment method, with most anticancer drugs used in chemotherapy exerting cytotoxic properties that inhibit cancer cell growth and proliferation. However, these drugs also harm healthy cells and tissues due to their harmful properties. To mitigate drug side effects and enhance their effectiveness, research is being conducted to develop more specific inhibitory compounds. Epidemiological and pathological studies suggest that cancers can be prevented or their progression halted.2 Dietary fruits and vegetables, in particular, have garnered significant attention due to their low toxicity and have been recognized as rich sources of chemopreventive compounds. Diets rich in fruits and vegetables have been shown to significantly reduce the risk of developing breast cancer, as well as lung, esophagus, larynx, pancreatic, colorectal, stomach, and prostate cancers.3 Broccoli (Brassica oleracea L. var. italica, Brassicacae) is an excellent source of isothiocyanates and is also abundant in vitamins (β-carotene, K, C), polyphenols, fatty acids, and dietary fiber. Various forms of broccoli, such as sprouts, powder, flour, fiber, florets, flakes, chips, etc., are gaining considerable attention today due to their preventive roles in non-communicable diseases like atherosclerosis, cancer (breast, prostate, lung, pancreas), and hypertension.4-7 In recent times, cell culture studies have become increasingly important, particularly in expediting cancer research, developing anticancer drugs, and assessing their effects. Nucleolar organizing regions (NORs) are loops of DNA containing ribosomal gene domains. As protein synthesis takes place in ribosomes within cells, ribosomes and the ribosomal RNA (rRNA) component are essential. The nucleolus houses extensive DNA loops, known as NORs, which transcribe RNA genes via RNA polymerase I. These loops of DNA are referred to as NORs. In normal cells, only a portion of rDNA is present in their nucleoli, but during rapid cell proliferation, there is an increased amount of rDNA within the nucleoli.8 Genetically active NORs can be stained using silver staining. NOR staining, also known as "silver staining," is a technique used to visualize and differentiate regions forming nucleoli at various stages of interphase or the cell cycle in various plant and animal tissues. When proteins within NORs are stained using the silver staining technique, they appear as dark brown-black spots within the cell nucleus.9,10 Previous studies have yielded positive results indicating that broccoli inhibits cancer progression by inducing apoptosis in cancer cells or arresting the cell cycle. Furthermore, there is currently no study in the literature investigating the antitumoral effects of broccoli on human breast cancer (MDA-MB-231) using AgNOR staining methods. In this study, we aimed to demonstrate the apoptotic effect of broccoli on MDA-MB-231 breast cancer cell lines and its potential impact on AgNOR protein synthesis. Additionally, we sought to determine the most appropriate dosage for cancer treatment.

Material Method

Preparation of Broccoli Extract: Three mature broccoli plants were procured from a local market in Kayseri for use in the study. On the day of acquisition, the broccoli was thoroughly washed and cut into pieces. Broccoli juice was then obtained by juicing the pieces using a juicer (Arçelik, K-1579, China). The resulting juice was collected in a glass jar and transported to the Erciyes University Anatomy Department Laboratory, where it was stored at a constant temperature of +4 ºC.

The samples were subsequently transferred to 50 ml centrifuge tubes. To remove the pulp, the samples were centrifuged four times for 15 minutes each at 5000 rpm using a centrifuge device (Selecta, Spain).

In order to eliminate macromolecules from the broccoli juice, it was first passed through a filter with a pore diameter of 0.45 µm and then through a filter with a pore diameter of 0.22 µm. Following this process, 28 ml of broccoli extract was obtained, which was then divided into four 7 ml beakers. The tops of the beakers were sealed with parafilm, and the extracts were stored in the dark at –20°C. After pretreatment, the frozen samples were placed in a lyophilizer (CHRIST) for drying. Lyophilization was conducted at -50 ºC under conditions of >0.065 mbar, lasting for 24 hours. The powdered broccoli was removed from the beaker and weighed on a precision balance (RADWAG) on aluminum foil. A total of 1.16 g of powdered broccoli was obtained from the broccoli juice. The resulting extract was stored at -20 ºC until further analysis.

Cell Culture: The MDA-MB-231 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Capricorn Scientific, CP21-4310) supplemented with streptomycin/penicillin (100 U/ml; Sigma Life Science, 046M4846V) and 15% fetal calf serum (FCS) (Biowest, S181G-500) in a humidified incubator (Sanyo, MCO-19 A/C(UV)) with an atmosphere of 5% CO2 at 37°C. Sterile conditions were strictly maintained to prevent the risk of contamination.

Healthy MDA-MB-231 cells were then divided into groups for broccoli treatment. Tissue culture plates with 24 wells, each containing 1000 µL of medium with 1×105 MDA-MB-231 cells, were used to determine the optimal broccoli dosage. These MDA-MB-231 cells were cultured in a humidified atmosphere containing 95% air and 5% CO2 overnight to allow attachment to the plates. After removing the medium, the MDA-MB-231 cells were washed three times with 500 µL of phosphate-buffered saline (PBS). Subsequently, the MDA-MB-231 cells were identified, and experimental groups were established using logarithmic concentrations of broccoli (125 and 250 µL/ml) applied to breast cancer cells after varying culture periods.

Cell Viability Assay and Proliferation: The cell suspension's concentration, expressed as cells per ml, was determined using the Trypan Blue cell counting method. To perform cell counting, a volume of the cell suspension was pipetted into an Eppendorf tube, and an equal volume of Trypan Blue solution was added. After 5 minutes of incubation, the mixture was placed between both sides of a closed Thoma slide (Marienfeld-Superior). Stained and unstained cells were counted using a microscope (Nikon Eclipse TS100). The average number of cells within the counting area was calculated, and the number of viable cells (cells/ml) was determined using the formula: Number of viable cells (cells/ml) = Average number of cells x 2x104.

Experimental Design: Experimental groups were established for Annexin V assays with 24-hour and 48-hour incubation periods, including a control group, as well as for cell cycle testing, which included the 125 µL/ml and 250 µL/ml broccoli treatment groups.

Annexin V Assay: Apoptosis analysis was conducted using the Muse Cell Analyzer device along with the compatible Muse Annexin V kit and dead cell assay reagent (Millipore; MCH100115).

MDA-MB-231 cells were seeded in 24-well plates at a density of 1x105 cells per well and allowed to incubate for 24 and 48 hours. Subsequently, the cells were treated with trypsin and stained with Annexin V and the dead cell reagent following the manufacturer's protocols (Millipore Corporation). The stained cells were then analyzed using the Muse Cell Analyzer (Millipore Corporation)

Cell Cycle Assay: The MuseR Cell Cycle Kit (Millipore; MCH100106) was employed to determine the cell cycle stage of the cells.

MDA-MB-231 cells were seeded in 24-well plates at a density of 1x106 cells per well and allowed to

incubate for 24 and 48 hours. Subsequently, the cells were treated with trypsin to detach them. Following detachment, the cells were stained with the MuseR Cell Cycle Kit, following the manufacturer's protocols (Millipore Corporation). The stained cells were then analyzed using the Muse Cell Analyzer (Millipore Corporation)

AgNOR Staining: MDA-MB-231 cells cultured in the presence of 125 µg/ml and 250 µg/ml broccoli, as well as control and treated groups, were spread onto clean slides and allowed to air dry at room temperature. After air drying, the slides were fixed in a fixative solution (3 volumes methyl alcohol:1 volume acetic acid), and the AgNOR staining method was conducted following a slightly modified version of the protocol as outlined by Lindler.11 The AgNOR-stained slides were examined using a light microscope (Leica DM 3000) and photographed with a digital camera (Imaging Color 12 BIT, Made in Canada).

The captured images of MDA-MB-231 cells were transferred to image processing software (ImageJ version 1.47t, National Institutes of Health, Bethesda, Maryland, USA), and both the total AgNOR area per nuclear area (TAA/NA) and mean AgNOR number were calculated using the 'freehand selection' tool for each nucleus. Fifty nuclei were assessed for each slide. An illustrative example of AgNOR staining of MDA-MB-231 cells is presented in Figure 1.

Statistical Analysis: The data's adherence to a normal distribution was assessed using the Shapiro-Wilk test, histograms, and q-q plots. One-way analysis of variance (ANOVA) was employed to statistically compare parameters across groups. The homogeneity of variances in the data was assessed using the Levene test. Multiple comparisons were performed using the Tukey test. Within each group, differences between the 24-hour and 48-hour measurements were assessed using the paired t-test. Data analysis was conducted using the Turcosa statistical software (Turcosa Analytics Ltd Co, Turkey, www.turcosa.com.tr). A p-value of <0.05 was considered statistically significant..

Results

Results

Annexin V and Dead Cell Test Results: In the graphs generated by the Muse device, four quadrants were formed by the intersection of the horizontal and vertical axes. Cell populations corresponding to different markers were observed in these quadrants. Dead cells were identified in the upper left quadrant, living cells in the lower left quadrant, late apoptotic cells in the upper right quadrant, and early apoptotic cells in the lower right quadrant. The data graphs obtained from the Muse Cell Analyzer device at the end of the 24 and 48-hour incubation periods are presented in Figure 2 and Figure 3.

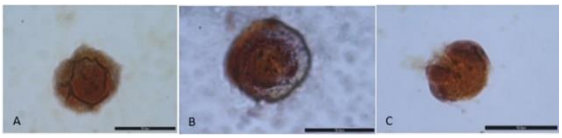

Figure 1. Demonstrative example of AgNOR staining of MDA-MB-231 cells. A: Control, B: 125 µl/ml broccoli, C: 250 µl/ml broccoli.

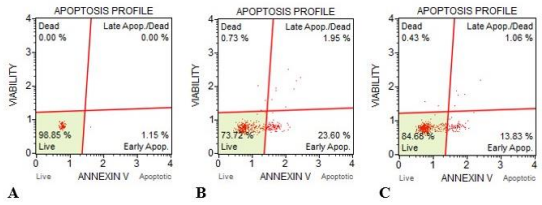

Figure 2. Data plots of 24-hour apoptosis rates by group on the Muse Cell Analyzer. A. Control group B. 125 µL broccoli extract, C. 250 µL broccoli extract.

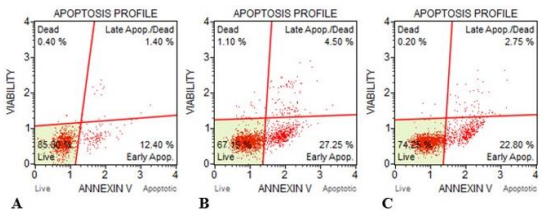

Figure 3. Data graphs of 48-hour apoptosis rates by groups on the Muse Cell Analyzer device. A. Control

group, B. 125 µl broccoli extract, C. 250 µl broccoli extract.

When the results related to viable cells were evaluated within the groups depending on the incubation period, a statistically significant difference was observed in the control group and the 250 µL/ml broccoli group ($p<0.05$), while no significant difference was found in the 125 µL/ml broccoli group. When the groups were evaluated based on the dose at 24 and 48 hours, a significant difference was found in all groups ($p<0.05$). Significant differences were also observed in the pairwise comparisons between the control group, 125 µL/ml, and 250 µL/ml groups in terms of viable cell counts at 24 and 48 hours ($p<0.001$). The most significant decrease in viable cell count was observed in the 125 µL/ml broccoli group.

There was no statistically significant difference in the number of early apoptotic cells when the results regarding early apoptotic cells were evaluated depending on the incubation period within the groups. However, a statistically significant difference was found in the number of early apoptotic cells when evaluated based on the dose at 24 and 48 hours ($p<0.05$). It was observed that the percentage of early apoptotic cells increased in the 125 and 250 µL/ml groups compared to the control group. According to multiple comparison tests, significant differences were found in the number of apoptotic cells at 24 hours between the control, 125 µL/ml, and 250 µL/ml groups ($p<0.001$). Based on these results, the 24-hour 125 µL/ml broccoli group exhibited the most significant increase in early apoptosis.

When the results regarding the percentage of late apoptotic cells were evaluated within the groups depending on the incubation period, a statistically significant difference was observed in the control and 125 µL/ml groups ($p<0.05$), while no significant difference was found in the 250 µL/ml group. When the groups were evaluated for the number of late apoptotic cells based on the dose, a significant difference was found at the 24-hour time point ($p<0.05$), but no significant difference was observed at the 48-hour time point. According to the multiple comparison test, the number of late apoptotic cells at 24 hours was significantly lower in the control group compared to the 250 µL/ml group ($p<0.05$).

When the total number of apoptotic cells was evaluated within the groups based on the dose, a significant difference was found at both the 24-hour and 48-hour time points ($p<0.05$). However, when the groups were evaluated depending on the incubation period, a significant difference was observed in the total number of apoptotic cells only in the control group ($p<0.05$), while no significant difference was found in the other groups ($p>0.05$). Multiple comparison tests revealed significant differences in the total number of apoptotic cells between all groups at 24 and 48 hours ($p<0.05$). The 125 µL/ml broccoli group exhibited the most significant increase in total apoptotic cells.

Cell Cycle Test Results: The data graphs obtained from the Muse Cell Analyzer device at the end of the 24 and 48-hour incubation periods are presented in Figure 4 and Figure 5.

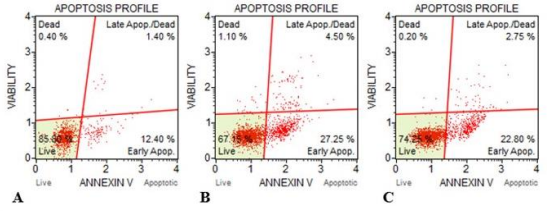

Figure 4. Data graphs of the percentage distribution ratios of the 24-hour cell cycle phases by groups, as read in the Muse Cell Analyzer device. A. Control group B. 125 µL broccoli extract C. 250 µL broccoli extract.

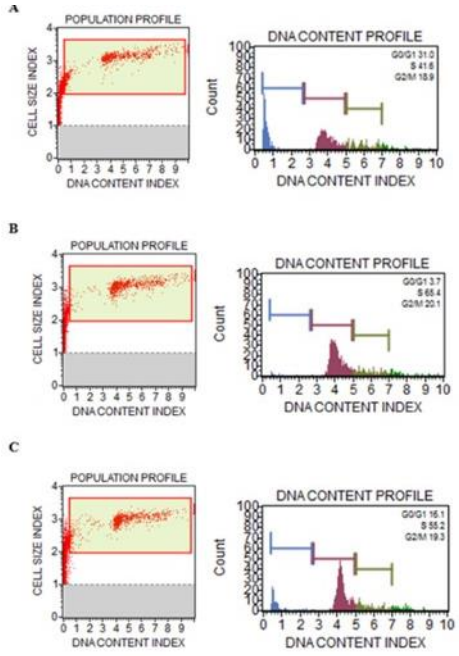

Figure 5. Data graphs of the percent distribution rates of 48-hour cell cycle phases by groups, as read in the Muse Cell Analyzer device. A. Control group B. 125 µL broccoli extract C. 250 µL broccoli extract.

When the results at 24 hours were evaluated, it was observed that the proportions of cells in the G0/G1 stage were lower in the 125 and 250 µL/ml groups compared to the control group ($p<0.05$), but there was no significant difference at 48 hours. When the groups were evaluated based on the dose, a significant difference was found in all groups at the G0/G1 stage ($p<0.05$) at both time points. Multiple comparison tests indicated significant differences in cell count measurements at 24 hours between all groups ($p<0.05$).

A significant dose-related difference was observed between the groups in the evaluation of cell cycles in the S stage at both the 24-hour and 48-hour time points ($p<0.05$). When the groups were evaluated based on the incubation period, a significant difference was found in all groups in the S phase at both time points ($p<0.05$). Multiple comparison tests revealed significant differences in S stage cell measurements at 24 and 48 hours between all groups ($p<0.05$).

In the evaluation of cell cycles at 24 and 48 hours, there was no significant dose-related difference between the groups. However, when the groups were evaluated based on the incubation period, a significant difference was observed in all groups in the G2/M stage ($p<0.05$).

AgNOR Results: The Total AgNOR Area/Nuclear Area (TAA/NA) ratio and mean AgNOR number were determined in the broccoli (125 and 250 µg/ml) groups and the control group.

As a result of the analysis, it was found that the AgNOR number (Table 1) and TAA/NA ratio (Table 2) significantly decreased at a dose of 125 µg/ml after 24 hours compared to the control group, and this difference was statistically significant ($p<0.05$) (Figure 6).

After 48 hours, the AgNOR number (Table 1) and TAA/NA ratio (Table 2) at a dose of 125 µg/ml also decreased compared to the control group and were found to be statistically significant ($p<0.05$) (Figure 7).

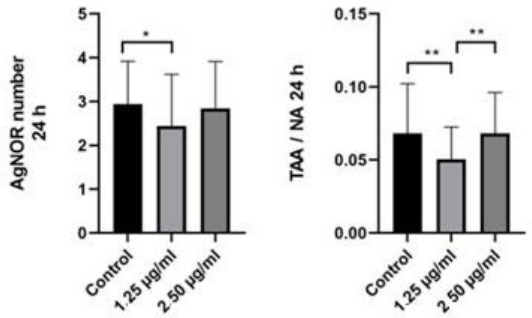

Figure 6. Comparison of AgNOR number and TAA/NA ratio between groups after 24 hours of incubation.

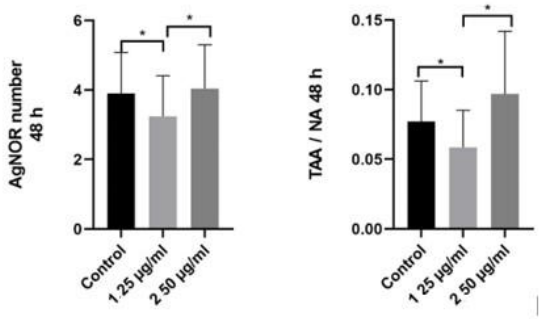

Figure 7. Comparison of AgNOR number and TAA/NA ratio between groups after 48 hours of incubation.

Table 1

Mean AgNOR number after 24 and 48 hours of incubation.

Hours/Groups	Control	125 µg/ml	250 µg/ml	*p*
24 hours	2,94±0,97[a]	2,44±1,18[b]	2,84±1,07[ab]	<0.001
48 hours	3,90±1,18[a]	3,24±1,17[b]	4,04±1,26[ab]	<0.001

AgNOR: Argyrophilic nucleolar organizer region

p<0.05 was considered statistically significant. Data are expressed as mean ± SD (Standard Deviation). Values with different letters (a and b) (p<0.05) in the same row at in-group measurement times were considered significantly different.

Table 2

TAA/NA value at the end of 24 and 48 hours of incubation.

Hours/Groups	Control	125 µg/ml	250 µg/ml	*p*
24 hours	**0,07±0,03[a]**	**0,05±0,02[b]**	**0,07±0,03[a]**	**<0.001**
48 hours	**0,08±0,03[a]**	**0,06±0,03[b]**	**0,09±0,04[a]**	**<0.001**

TAA/NA:Total AgNOR area/Nuclear area

p<0.05 was considered statistically significant. Data are expressed as mean ± SD (Standard deviation). Values having different letters (a and b) (p<0.05) on the same row at in-group measurement times were considered to be significantly different.

Discussion

Cancer treatment methods, including surgery, radiotherapy, hormone therapy, chemotherapy, and immunotherapy, often come with significant side effects. Therefore, there is a growing need to identify safe and potent anti-cancer compounds from natural sources.[12]

Epidemiological studies have consistently shown that a diet rich in fruits and vegetables can significantly reduce the risk of various types of cancer, including breast cancer.[13,14] Vegetables from the Brassicaceae family, such as broccoli, are particularly rich in chemopreventive compounds. Phytochemicals and their precursors found in these vegetables have demonstrated the ability to reduce the risk of cancer development.[15]

One such compound found in broccoli is sulforaphane (SFN), which has been extensively studied for its anti-cancer properties. SFN can inhibit phase I enzymes responsible for converting procarcinogens into carcinogens, and it can stimulate phase II enzymes, aiding in the detoxification and removal of carcinogens from the body. SFN has shown promise in preventing cancer initiation and progression by blocking the cell cycle or inducing apoptosis.[16]

Numerous studies have investigated the anti-cancer effects of SFN, often isolated from broccoli and other cruciferous vegetables, in various cancer types, including melanoma, prostate cancer, and pancreatic cancer. SFN has been shown to inhibit cell viability, induce apoptosis through the activation of genes like caspase 3 and 9, and interfere with signaling pathways such as NF-κB. It has also demonstrated the ability to overcome resistance to chemotherapy drugs like doxorubicin.[18,19] Oral or intraperitoneal administration of SFN inhibited tumor growth in prostate PC-3 and pancreatic Panc-1 xenografts.[20] It has been shown that the risk of premenopausal breast cancer is

inversely proportional to broccoli consumption.[15] Oral SFN reached the mammary gland and increased the detoxification enzyme activity.[21] By interfering with NF-κB-induced anti-apoptotic signaling, SFN has demonstrated the efficacy of abolishing pancreatic tumor resistance to TNF-related apoptosis-inducing ligand (TRAIL).[22,23] Another study showed that SFN can overcome doxorubicin resistance and restore apoptosis induction in cells.[24,25] These findings provide a strong rationale for investigating the chemopreventive property of SFN, broccoli, and broccoli sprouts in clinical trials.

Licznerska et al.[24] showed that MDA-MB-231 and MCF-7 breast cancer cell lines induced apoptosis after 72 hours of treatment at 5 and 20 µM SFN concentrations.

Lewinska et al.[25] stimulation of breast cancer cells (MDA-MB-231, SK-BR-3 and MCF-7) with low doses of SFN (5-10 µM) inhibits cell cycle, increases p21 and p27 levels and promotes cellular senescence, 20 µM It has shown that apoptosis is induced at this concentration.

Licznerska et al.[26] evaluated the effect of R-SFN on phase II enzyme induction and expression of AhR, Nrf2 and Erα, NQ01 in breast cell lines. Induction of NQO1 by R-SFN has been found to support treatment with certain chemotherapeutics in MDA-MB-231, while in non-tumorigenic cells representing the early stage of breast carcinogenesis and partially MCF7 cells, R-SFN protects against cancer initiation and progression.

The present study evaluated the effects of SFN and broccoli extracts on breast cancer cells, specifically MDA-MB-231 cells. Previous research has indicated that low doses of SFN can induce apoptosis, inhibit the cell cycle, increase levels of proteins like p21 and p27, and promote cellular senescence in breast cancer cells. SFN has also been explored as a potential histone deacetylase (HDAC) inhibitor in breast cancer, showing inhibitory effects on cell growth and proliferation. It reduced the expression of key proteins involved in breast cancer proliferation. These results support in vivo testing of SFN and warrant future studies examining the clinical potential of SFN in human breast. One of the recent studies has shown that 5, 25 and 100 µm ITC cause cytotoxic effects (reduction in viable cell count) in MCF-7 cells in vitro by altering DNA damage and repair-associated proteins.[27]

Pasko et al.[28] in a study comparing the apoptotic effect of broccoli sprouts and flowers, obtained broccoli (5 g) extracts by freeze-drying and dissolved the powdered broccoli in DMSO. In BJ, SW480, HepG2 cells 0.25, 1 and 2.5 mg/ml broccoli were incubated for 24 hours and Annexin V test was performed. It was found that the sprouts were more effective than the flowers and the 2.5 mg/ml broccoli dose had a significant effect on apoptosis on colorectal cancer cells. The doses of broccoli powder extracts whose apoptotic effects against MDA-MB-231 cell will be examined. The study was determined according to the pilot study conducted with reference to the study. When the literature was evaluated, SFN, which is the active ingredient of broccoli in cell culture

studies, was determined using high performance liquid chromatography (HPLC). However, SFN could not be determined in this study due to lack of equipment. Broccoli extracts were dried and powdered and dissolved in the medium and given to the cells. For this reason, it is thought that the result obtained is due to the bioactive components in broccoli, especially SFN. Since pure SFN cannot be used in basic nutrition, it is thought that this effect should be achieved by naturally fortifying cruciferous vegetables.

The effects of many herbal extracts on cancer cell lines are being investigated. In such studies, it has been shown that the percentage of total apoptotic cells increases as a result of Annexin V analysis[29,30] In this study, MDA-MB-231 cells were cultured for 24 and 48 hours at 37^0C and 5% CO_2, and the parameters obtained using Annexin V and cell cycle test were evaluated to determine the percentage of apoptosis. After 24 and 48 hours, it was found that the percentage of apoptosis on MDA-MB-231 cells increased and there was a significant decrease in the percentage of viable cells ($p<0.05$).

A significant increase was observed in the % total apoptosis rate in the MDA-MB-231 cell line cultured with broccoli compared to the control group. However, the increase was not significant over time. The percentages of early apoptosis rate were significantly increased in all groups compared to the control group. However, no statistically significant results were found depending on time. Late apoptotic responses were lowest in the 24-hour control group ($p<0.05$).

Stopping phases in the cell cycle can inhibit the proliferation of cancerous cells. This was realized by some phytochemical products and significant results were found.[31]

In an other study that investigated the effects of SFN on BALB/c mouse kidney cancer cells in vivo and in vitro, 15 µM SFN inhibited the cell cycle in the early phase of G2M (prophase/prometaphase) in 24 hours in the cell cycle test.[31]

Cheng et al.[32] were determined that in vitro study in which ZR-75-1 cells were treated with different concentrations of SFN (0, 6.25, 12.5, and 25 µM) for 24-72 hours, SFN after 24 hours, It has been shown to cause an increase in the G1 phase cell population, resulting in a delay in the G1/S phase checkpoint of ZR-75-1 cells. This result showed that SFN increased cell populations in the G1/S phase while simultaneously decreasing the S phase population.

Rutz J. et al.[33] in his study, it was observed that 5 µM SFN exposure increased the number of cells in the S phase of the cell cycle in Caki-1 and A498 cells and decreased the number of cells in the G0/G1 phase. When Caki1 cells were treated with 20 µM SFN, it was observed that the number of cells increased in both S and G2/M stages.

Experiments on osteosarcoma cells showed that SFN was effective in the G2/M phase at concentrations of ≤10 µM, while it was effective in the S phase at concentrations of >10 µM.[34]

Previous studies have shown that SFN can induce cancer cell apoptosis and cell cycle arrest.[31-35] In the current study, the 24th and 48th hour results showed a statistically

significant increase in cell count in the S phase of the broccoli extract ($p<0.05$). The most significant increase was seen in the number of cells in the S phase. This suggested that broccoli extract could effectively stop cancer cells in S phase. Rutz et al.[33] observed the effect of SFN on the cell cycle in ZR-75 human breast cancer cells and observed an increase in the number of cells in the S phase and G2M phase. The results of this study support the present study.

In the G0/G1 phase, there was a significant decrease in the groups containing broccoli extract compared to the control group ($p<0.05$). While the most significant decrease in the number of cells in the G0/G1 phase was observed in the group containing 125 µl/ml broccoli extract, the most significant increase in the number of cells in the S phase was observed in the 125 µl/ml broccoli extract groups. In the G0/G1 phase, the cells are stopped at the G1 to S checkpoint transition by suppressing the function of the complex formed by Cyclin E and Cyclin Dependent Kinase -2, which are the main cell cycle regulators, to promote cell transition from the G1 phase to the S checkpoint.[35] Based on this, it was thought that broccoli extracts slowed down the cell cycle at the G1 to S checkpoint transition in MDA-MB-231 cells. A significant difference was found in the 125 µg/ml broccoli extract group after 24 and 48 hours of incubation in AgNOR staining ($p<0.05$).

During the interphase, NORs are associated with a great number of regulatory proteins and they have roles as functional subunits of the nucleolus. Alterations in AgNOR protein amounts also reflect the metabolic activities of the cells. We performed various numbers of studies on malign and benign lesions.[36,37] In these studies, we evaluated mean AgNOR number and TAA/NA ratio as a new approach that may contribute to routine cytopathology for determining the proliferation activity of cells in malignant and benign lesions. In the current study, we aimed to identify whether broccoli has an effect on cell proliferation and whether the detection of AgNOR protein amounts may be used to detect the therapeutic benefits of the drugs and new metabolites that have a potential to be used in cancer treatments. The current study showed that the expression capacity of rRNA gene, as detected via total TAA/NA and/or AgNOR number per total nuclear number, decreased 125 µg/ml broccoli extract group. It may be said that broccoli has an important role in prevention of tumor formation and triggers or supresses the synthesis of some other proteins that have important features and functions in signaling the transduction pathways and gene expression regulation in tumor cells.

The study involved assessing apoptosis and cell cycle progression in MDA-MB-231 cells cultured with broccoli extract. Results showed a significant increase in apoptosis and a decrease in viable cells, with early apoptosis being most prominent in the group treated with 125 µl/ml broccoli extract at 24 hours.

Cell cycle arrest is another mechanism by which anti-cancer compounds can inhibit the proliferation of cancer cells. The current study observed that broccoli extract effectively arrested MDA-MB-231 cells in the S phase of the cell cycle. This

suggests that broccoli extract may be effective at halting cancer cell progression.

In summary, this research contributes to the expanding body of evidence that highlights the potential anti-cancer properties of SFN and broccoli extracts. Although this study did not pinpoint the exact mechanism of action or identify the specific active ingredient responsible for these effects, the findings strongly indicate that including broccoli and its bioactive components in one's diet could offer protection against breast cancer. Further investigations, including clinical trials, are necessary to delve deeper into the chemopreventive qualities of broccoli and its extracts.

The most important limitation of the study is; the content analysis of broccoli cannot be performed and the effective dose of its active ingredient, SFN, cannot be shown. In addition, it is not known under what conditions the broccoli used was stored until it was obtained.

Conclusion

Numerous studies have provided compelling evidence supporting broccoli as a potential anticancer agent, demonstrating its ability to inhibit the proliferation of various cancer cell lines, including those associated with the liver, stomach, colon, bladder, kidney, breast, and prostate. This specific study focused on evaluating the effects of powdered broccoli extracts obtained from different concentrations of broccoli juice on MDA-MB-231 cells.

The results of the study revealed that the broccoli extract led to a decrease in the number of viable cells, a significant increase in both early and total apoptosis, and a deceleration of the cell cycle during the transition from the G1 to S checkpoint ($p<0.05$). These findings align with prior research on apoptosis, suggesting that broccoli induces apoptosis in cancer cells through a variety of mechanisms, with the most pronounced effect observed in the 125 µL/ml broccoli group. Furthermore, the study demonstrated a reduction in the synthesis capacity of AgNOR proteins in the 125 µL/ml broccoli group, implying that broccoli may contain essential molecules for cancer prevention. Additionally, the research proposed that assessing the TAA/NA ratio could serve as a biomarker to evaluate the efficacy of therapeutic approaches and determine the most suitable dosage for cancer treatment.

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