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Effect on MDA-MB231 human breast cancer cell line treated with bilberry (*Vaccinium myrtillus*) using Annexin V and AgNOR staining

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Ethics Committee Approval
This study is a cell culture study that is outside the scope of studies requiring ethical approval.

Conflict of Interest
No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Cancer has become a prevalent disease, emerging as one of the major chronic health issues today. Currently, common treatments against cancer include chemotherapy, radiotherapy, surgery, and the use of chemically synthesized drugs. However, despite significant advancements in diagnostic methods and treatments, drug resistance and metastasis remain primary hurdles to successful cancer therapy. Consequently, attention has been shifted towards exploring alternative treatments and therapies against cancer. This study sought to examine the time and dose-dependent effects of blueberry (*Vaccinium myrtillus* L) on MDA-MB-231 cell lines.

Methods: The study used the MDA-MB231 breast cancer cell line. We established three groups: control, 40 µl/ml bilberry, and 80 µl/ml bilberry, which were incubated at 37°C and 5% CO_2 for 24 and 48 h, respectively. After incubation, we examined the viability, apoptosis, and cell cycle of MDA-MB-231 cells with the Muse Cell Analyzer and assessed the status of nucleolar organizer region (NOR) proteins via silver nitrate (AgNOR) staining.

Results: Bilberry extracts were found to enhance apoptosis and exhibit a cytotoxic effect, thereby reducing cell proliferation in MDA-MB-231 cells after 24 and 48 h of culture. There was notably increased apoptosis at concentrations of 40 µl and 80 µl. Moreover, after 48 h of incubation, a significant difference emerged between the control and 40 µg/ml bilberry samples, notably in the average AgNOR count and the total AgNOR area/total nuclear area ratio.

Conclusion: Our study suggests that blueberries may be a potential therapeutic candidate for cancer treatment, thereby potentially enriching cancer research.

Keywords: MDA-MB-231, bilberry, apoptosis, *in vitro*

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Introduction

Despite significant advancements in cancer diagnosis and treatment, drug resistance and metastasis remain primary challenges. Current cancer treatments include chemotherapy, radiotherapy, and chemical drugs [1]. Although these treatments form traditional therapy procedures, plant-derived compounds account for a significant portion of today's pharmaceuticals, owing to their recognized medical importance in oncology [2]. Consequently, alternate cancer treatments and therapies are gaining prominence.

Epidemiological studies indicate lower cancer incidence rates within populations consuming high volumes of plant-based foods. Herbal medicines have been primary treatment sources for several years and remain in employment [3]. Research into the development of plant-based anticancer agents has surged since the 1950s [4]. Interestingly, around 75% of present-day anticancer agents are plant-derived [5].

Ingestion of fruits and vegetables rich in bioactive phytochemicals contributes notably to reducing the prevalence of cancer and other chronic diseases [6]. Research suggests that the anticancer properties of plants can be attributed to the phenolic compounds they contain. Both monophenolic and polyphenolic compounds in plant foods have demonstrated the potential to suppress or mitigate the onset, growth, and spread of cancer, both *in vivo* and *in vitro* [7].

The fruit and leaves of bilberries (*Vaccinium myrtillus*: VM) are abundant in phenolic compounds, including flavonoids [8]. Babova found that bilberry fruit houses five of the six natural anthocyanins also found in other fruits [9]. Seeram [10] identified bioactive food components, specifically in berry fruits, that showed potential in cancer prevention, as demonstrated by various preclinical studies.

Berries such as blackberries, blueberries, and strawberries, as well as their bioactive components, have been studied for their anticancer activities [11]. While most preclinical research centered on cancer prevention using berries targeted specific cancers like the esophagus [12-13], colon [14], lung [15], and skin [16], some studies found no significant benefits. That being said, blueberries (from VM), which are abundant in phenolic compounds and high in antioxidants, have shown promise in protecting against chronic diseases. These include cardio and cerebrovascular diseases, atherosclerosis, diabetes, and even cancer. Findings suggest that the active components in blueberries might work as effective anticancer agents, both as functional foods and nutritional supplements [17-18]. This study aims to explore the potential effects of VM treatment on NOR protein synthesis and its potential apoptotic effect on the MDA-MB-231 cell line.

Materials and methods

Preparation of *Vaccinium myrtillus* extract

We obtained the blueberries for this study from Artvin province in July, coinciding with their harvest time. We extracted juice using a juicer and transferred it to 50ml centrifuge tubes. Upon centrifuging the juice at 5500/min at 15°C for 30 min with a Selecta machine from Spain, we passed it through 0.45-µm and then 0.22-µm filters. We then froze the filtered juice into 7ml units, which we dried with a lyophilizer (Christ Freeze Dryer-Alpha 1-2 LD) to form a powder. We stored the bilberry extracts at -20°C until usage.

Cell culture

The MDA-MB-231 cell line, obtained from the American Type Culture Collection (Manassas, VA, USA), was cultured in Dulbecco's modified Eagles medium (DMEM, Capricorn Scientific, CP21-4310). This included streptomycin/penicillin (100 U/ml; Sigma Life Science, 046M4846V) and 15% fetal calf serum (FCS, Biowest, S181G-500) in a humidified 5% CO_2 atmosphere at 37°C (Sanyo, MCO-19 A/C(UV)). Healthy MDA-MB-231 cells were then divided into groups and subjected to a broccoli treatment. A tissue culture plate with 24 wells, each containing 1000 µL of medium and 1×10^5 MDA-MB-231 cells, was then utilized to determine the optimal VM dose. The cells were rinsed three times in 500 µl phosphate-buffered saline (PBS) after the medium was removed. Finally, experimental groups were formed by setting log concentrations of VM (40 and 80 µl/ml VM) on the breast cancer cells.

Cell viability assay and proliferation

We used the Trypan Blue cell counting method to determine the number of cells in each ml of the cell suspension. A portion of this cell suspension was mixed with an equal proportion of Trypan Blue solution in an Eppendorf tube. After a 5-min incubation period, the coverslip was shifted to both sides of the sealed Thoma slide (Marienfeld-Superior). We then differentiated and counted the stained and unstained cells under a microscope (Nikon Eclipse TS100).

Experimental design

For cell cycle testing, groups were created for 24 and 48-h incubations using Annexin V, along with 40 and 80 µl/ml VM control groups.

Annexin V assay

We conducted an apoptosis analysis using the Muse Cell Analyzer device alongside the compatible Muse Annexin V kit and dead cell assay reagent (Millipore; MCH100115). MDA-MB-231 cells were grown in 24-well plates, each containing 1x105 cells, and incubated for 24 and 48 h. Afterward, the cells were treated with trypsin and stained using Annexin V and a dead cell reagent, as per the manufacturer's guidelines. The stained cells were then analyzed with the Muse Cell Analyzer (Millipore Corporation).

Cell cycle assay

The MuseR Cell Cycle Kit (Millipore; MCH100106) was utilized to identify the cell cycle stage of MDA-MB-231 cells. These cells were grown in 24-well plates, with 1×10^6 cells per well, and were incubated for 24 and 48 h. Following this, the cells were treated with trypsin for removal. Subsequently, they were stained using the MuseR Cell Cycle Kit, per the manufacturer's protocols (Millipore Corporation), and analyzed using the Muse Cell Analyzer (Millipore Corporation).

AgNOR staining

MDA-MB-231 cells were cultured with a control, 40 µg/ml, and 80 µg/ml VM. They were then spread on a clean slide and allowed to dry at room temperature. After air drying, the slides were treated with fixative (a mixture of 3 volumes of methyl alcohol and 1 volume of acetic acid). These slides, once stained with silver nitrate (AgNOR), were examined and photographed

using a Leica DM 3000 light microscope and a Canadian-made Imaging Color 12 BIT digital camera. The images of MDA-MB-231 cells were transferred to ImageJ version 1.47 image processing software (National Institutes of Health, Bethesda, Maryland, USA) for further study. The total AgNOR area and the average AgNOR number per nuclear area were calculated by examining cell nuclei with the "freehand selections" tool.

Statistical analysis

The study evaluated the normal distribution using Shapiro-Wilk's test statistic, a histogram, and a q-q graph. Groups were compared using a one-way analysis of variance (ANOVA). Variance homogeneity was assessed using Levene's test. The differences between 24-h and 48-h measurements in each group were analyzed using a paired t-test. Tukey's test facilitated multiple comparisons. Per Tukey's test, if the alphabetic superscripts contain the same letter, it indicates no significant difference between the groups. Conversely, a difference implies statistical significance. Data were reported as means and standard deviations. Analysis was performed using TURCOSA statistical software (Turcosa Analytics Ltd Co, Turkey, www.turcosa.com.tr), with a significance level set at $P<0.05$.

Results

Annexin V findings

A significant difference was observed in the number of viable cells between the 24-h and 48-h measurements in the control group, as well as the 40 µl and 80 µl VM extract groups ($P<0.001$) (Figure 1). After 24 h of culture, both the 40 µl and 80 µl VM extract groups displayed significantly more viable cells than the control group ($P<0.001$). This trend continued after 48 h, with both VM extract groups maintaining significantly more viable cells than the control ($P<0.001$). However, there was no significant difference observed between the 40 µl and 80 µl VM extract groups (Table 1).

Figure 1: Annexin V test results.

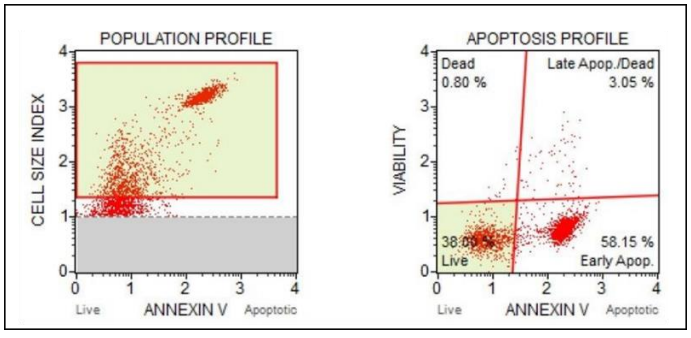

Table 1: In the Annexin V & Dead and live cells after 24 and 48 h.

Groups	Live Cell 24 h Mean (SD)	Live Cell 48 h Mean (SD)	*P*-value *
Control	98.62(0.63)a	84.81(0.39)b	<0.001
40 µl 40 µl VM	85.3(2.30)a	39.4(1.3)b	0.002
80 µl 80 µl VM	85.20(2.1)a	38.0(1.7)b	<0.001
***P*-value #**	<0.001	<0.001	

SD: standard deviation, *P**: One-way analysis of variance, *P*#: Paired t-test, VM: *Vaccinium myrtillus* L.

Cell cycle findings

G0/G1 findings: Upon evaluation of the G0/G1 measurement, a significant difference between all groups were identified based on both the 24 and 48-h results ($P<0.001$). The cell proportions in the G0/G1 stage were notably higher in all other groups, specifically the 40 µl VM extract and 80 µl bilberry groups, as compared to the control group ($P<0.001$). A significant difference was also identified between the 40 and 80 µl/ml VM extract doses when the 24 and 48-h results were considered. The 80 µl/ml VM extract was significantly higher compared to the 40 µl/ml VM group ($P<0.001$) (Table 2).

Table 2: G0/G1 measurement after 24 and 48 h in cell cycle test.

Groups	G0/G1 24 h Mean (SD)	G0/G1 48 h Mean (SD)	*P*-value *
Control	18.2(0.30)a	76.00(1.10)a	<0.001
40 µl 40 µl VM	21.6(0.4)b	91.2(0.3)b	<0.001
80 µl 80 µl VM	25.9(1.2)c	94.1(0.8)c	<0.001
***P*-value #**	<0.001	<0.001	

SD: standard deviation, *P**: One-way analysis of variance, *P*#: Paired t-test, VM: *Vaccinium myrtillus* L.

S measurement: A significant difference was observed between the 24-h and 48-h S measurements in the control group and the 40 and 80 µl/ml VM extract groups ($P<0.001$). The S measurement was higher than that of the control group, as well as the 40 and 80 µl/ml VM extract groups at both 24 and 48 h ($P<0.001$). A comparison of the 40 and 80 µl/ml VM groups revealed that the 80 µl/ml group recorded a lower measurement than the 40 µl/ml group ($P<0.001$). After 48 h, both the 40 and 80 µl/ml VM groups showed a significant reduction compared to the control group, with the decrease being more pronounced in the 80 µl/ml VM group ($P<0.001$) (Table 3).

Table 3: S measurement after 24 and 48 h in the cell cycle test.

Groups	S 24 h Mean (SD)	S 48 h Mean (SD)	*P*-value *
Control	44.90(0.2)a	12.3(0.1)b	<0.001
40 µl 40 µl VM	41.3(0.4)a	3.3(0.7)b	<0.001
80 µl 80 µl VM	39.4(0.3)a	1.8(0.2)b	<0.001
***P*-value #**	<0.001	<0.001	

SD: standard deviation, *P**: One-way analysis of variance, *P*#: Paired t-test, VM: *Vaccinium myrtillus* L.

G2/M measurement: Upon evaluating the G2/M measurement, a substantial decrease was noted in the 80 µl/ml VM group in comparison to both the control and 40 µl/ml VM groups after a 24-h culture period ($P<0.001$). A further statistically significant decline was seen in both the 40 and 80 µl/ml VM groups compared to the control after 48 h of culturing ($P<0.001$) (Table 4).

Table 4: G2/M measurement after 24 and 48 h in cell cycle test.

Groups	G2/M 24 h Mean (SD)	G2/M 48 h Mean (SD)	*P*-value *
Control	22.2(0.4)a	6.0(0.3)a	<0.001
40 µl 40 µl VM	22.5(0.9)a	2.4(0.2)b	<0.001
80 µl 80 µl VM	19.2(0.4)a	2.8(0.5)b	<0.001
***P*-value #**	<0.001	<0.001	

SD: standard deviation, P*: One-way analysis of variance, P#:Paired t-test, VM: *Vaccinium myrtillus* L.

AgNOR staining results: After 24 h of incubation, all VM groups showed no significant differences compared to the control group. However, after 48 h, there was a significant reduction in the mean AgNOR number in the 40 µg/ml VM group compared to the control group ($P<0.001$) (Table 5).

Table 5: Mean AgNOR number after 24 and 48 h of incubation.

H/Groups	Control	40 µg/ml VM	80 µg/ml VM	*P*-value
24 h Mean (SD)	3.78(1.09)	3.90(1.24)	3.96(1.19)	>0.05
48 h Mean (SD)	4.04(1.23)a	3.42(1.25)b	3.70(1.16)ab	<0.001

SD: standard deviation, AgNOR: Argyrophilic nucleolar organizer region, VM: *Vaccinium myrtillus* L.

Upon examining the 24-h incubation data, the TAA/NA value was not statistically significant. However, for the 48-h incubation data, this value significantly decreased ($P<0.001$) in the 40 µg/ml VM groups compared to the control group (Table 6).

Table 6: TAA/NA value at the end of 24 and 48 h of incubation.

H/Groups	Control	40 µg/ml VM	80 µg/ml VM	*P*-value
24 h Mean (SD)	0.05(0.03)	0.06(0.02)	0.06(0.02)	>0.05
48 h Mean (SD)	$0.08(0.03)^{a}$	$0.06(0.02)^{b}$	$0.07(0.03)^{ab}$	<0.001

SD: standard deviation, TAA/NA: Total AgNOR area (TAA)/Total nuclear area (NA) ratio, VM: *Vaccinium myrtillus* L.

Discussion

VM holds special significance among fresh fruits and vegetables due to its abundant antioxidants, anthocyanin levels, and other phenolic compounds [19]. It also provides a rich source of phenolic compounds like chlorogenic acid, quercetin, campherol, myricetin, procyanidin, catechin, epicatechin, and resveratrol. These compounds contribute to VM's antioxidant activity, and they are also rich in vitamin C [20].

Many studies have demonstrated the effects of VM, showing its anticancer properties, antioxidative potential, and antimicrobial and anti-inflammatory activities. There is also evidence to suggest its potential application in treating various diseases through clinical studies. VM appears to play a significant role in mitigating certain types of cancers, eye diseases, neurological disorders, and diabetes. Many of these studies opted to use extracted anthocyanins as opposed to direct VM juice. As such, anthocyanins are seen to contribute significantly to the VM plant's efficacy [21-23].

There's substantial evidence that VM hinders cancer's growth and spread. Studies suggest one way VM influences cancer is by prompting apoptosis in cancer cells, thereby inhibiting their growth. Zhao et al. [24] researched the potential anticancer effects of VM extracts loaded with anthocyanin against colon cancer. Their findings indicated that these extracts suppressed the growth of HT-29 cancer cells, demonstrating antitumor effects. In a 2008 study, Aiyer et al. [25] demonstrated that mice orally administered with whole VM powder experienced a 40% reduction in mammary tumor volume compared to the control group animals. Earlier studies corroborate that a diet rich in fruits and vegetables is linked with a decreased cancer risk [26].

Research findings support the antitumor effects of VM, warranting further investigation. Faria et al. [27] tested an anthocyanin bilberry extract and an anthocyanin-pyruvic acid adjunct extract on MDA-MB-231 and MCF7 cancer cell lines. Both extracts significantly reduced cell proliferation at 250 µg/mL after 24 h of incubation, with the adjunct extract manifesting a stronger effect on MDA-MB-231 cells. This suggests an estrogen receptor-independent activity. These findings confirm the efficacy of VM anthocyanins and related anthocyanin-pyruvic acid adducts as anticancer agents, inhibiting cancer cell proliferation [27].

Our findings align with these previous studies. We concluded that DNA synthesis was significantly inhibited after 48 h of treatment with 80 µl/ml VM extract. We observed that both 40 and 80 µl/ml VM extract doses slowed or stopped cell mitosis within 48 h in MDA-MB-231 cells.

Adams et al. [28] found that MDA-MB-231 human breast cancer cells treated with VM extract (12.5–25 µL/mL) for 72 h exhibited 1.5 times more cell apoptosis than the control group. In our study, we noticed an increased apoptotic response in cells treated with 40 and 80 µl VM extracts over 24 and 48 h.

The cytotoxic effects of this VM extract on MDA-MB-231 cells were evaluated in our study, and the doses were determined based on a pilot study [28].

Our study concluded that the application of 40 µl and 80 µl of bilberry extracts heightened apoptosis, exerted a cytotoxic effect, and hindered cell proliferation in MDA-MB-231 cells after 24 and 48 h of culture. Correspondingly, apoptosis increased in the 40 and 80 µl/ml VM groups after the same duration. A considerable decrease in viable cells was observed in both VM groups compared to the control group, showcasing the cytotoxic impact of bilberry on MDA-MB-231 cells.

Analysis of G0/G1 measurement results revealed minimal activity in the VM groups at the end of 24 and 48 h. Based on S phase data, DNA synthesis significantly diminished or halted in the 80 µl/ml VM group after 48 h. Evaluation of the G2/M phase results showed no evident effectiveness of the 40 µl/ml VM group after 24-h culture. Conversely, the 80µl/ml VM group showed a slowdown in mitotic division. Notably, a significant drop was recorded in the 40 and 80 µl/ml VM groups compared to the control group after 48 h. Hence, both doses - 40 and 80 µl/ml VM - effectively suppressed or halted mitosis.

Limitations

A limitation of this study is that it cannot analyze the anticarcinogenic properties of active substances in blueberries using cancer cell culture.

Conclusions

Our research found that VM augments apoptosis in MDA-MB-231 human breast cancer cells by various mechanisms, consistent with other studies on VM. We also found that the AgNOR number increases after 48 h of incubation at a 40 µl/ml VM dose. These findings suggest the potential use of AgNOR as a biomarker for determining the therapeutic dosage and highlight VM as a potential candidate for use against cancer. Additionally, incorporating blueberries into dietary cancer prevention strategies may be beneficial. Future *in vitro* studies and further investigations into how blueberries modulate the metastatic potential of MDA-MB-231 cells, currently being studied in vivo, will enrich the related literature.

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