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Anticancer and Antibacterial Effects of Blueberry Fruit (Vaccinium corymbosum L.) in Three Developmental Stages

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ABSTRACT

Background and Aim: Blueberries, scientifically known as *Vaccinium corymbosum* L., contain various secondary metabolites such as flavonoids and phenolic acids. These compounds are recognized for their potential in preventing cancer and antimicrobial properties.

Materials and Methods: The extract's antibacterial activity was assessed using the time-killing assay, while its anticancer activity was evaluated using the MTT assay.

Results and Conclusion: The findings from the antibacterial tests revealed that the fruit extracts exhibited minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at concentrations of 3.5-4.2 mg/mL and 5.6-5.9 mg/mL, respectively. Furthermore, the MTT assay demonstrated that the blueberry extract displayed cytotoxic effects on cancer cell lines (HeLa and MCF7), while it showed no significant cytotoxicity on normal cells (L929). Treatment with the unripe blueberry fruit extract significantly decreased the viability of cancer cells compared to the other extracts. Specifically, the highest level of cellular toxicity among all extracts was observed in the HeLa cancer cell line at a concentration of 1.5 mg/L after 72 hours (more than 90%). Based on these findings, it can be concluded that blueberry fruit extract possesses enhanced antibacterial and anticancer properties. Moreover, this study indicated that the early stages of fruit growth exhibit greater cytotoxic effects against cancer and bacterial cells. These results significantly affect the food, pharmaceutical, and plant biotechnology industries.

Keywords: HCMV, Human Cytomegalovirus, Iran, Molecular Prevalence, Prostate Neoplasm, Prostate Cancer

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1. Introduction

Blueberries, specifically the reddish-purple or dark blue variety (*Vaccinium corymbosum* L.), grow on deciduous shrubs belonging to the Ericaceae family, with an average diameter of 1.5 cm. Berries are rich in secondary metabolites, which are synthesized during the fruit ripening process and can play a significant role in seed dispersal through pigments and flavor compounds. Moreover, these secondary compounds contribute to the protection of developing fruits against diseases **(1)**.

Currently, cancer is one of the major causes of death worldwide, resulting from various factors, including mutagens and carcinogenic chemicals in the environment **(2)**. Cervical cancer is the second leading cause of cancer-related deaths among women, with an estimated 280,000 deaths occurring in developing countries. Cervical cancer competes with adjacent tissues for blood supply and destroys them **(3)**. Furthermore, breast cancer is the most common cancer in women over 40 years old, accounting for 33% of all female cancers. The prevalence of this cancer is increasing, and it has become the most prevalent malignancy among women **(4)**.

Research has shown that blueberry can reduce the proliferation of cancer cells, thus preventing cancer progression and cardiovascular diseases **(5)**. Additionally, blueberries contain phenolic acids and flavonoids with biological activities such as radical scavenging and antimicrobial properties **(1)**.

Salmonella is a foodborne pathogen that is transmitted to humans through various food sources, including contaminated poultry, eggs, dairy products, and fresh vegetables **(6)**.

Currently, research is focused on inhibiting antibiotic-resistant bacteria using natural compounds. The antimicrobial properties of bilberry, raspberry, blackberry, and especially blueberry among different berries are remarkable **(7)**. Other studies have found that a minimum inhibitory concentration of 450 mg/mL of Elliot blueberry extract inhibited *Salmonella enteritidis* growth **(8)**.

Fruits' antibacterial and anticancer properties have been confirmed throughout the ripening process. Information about changes in secondary metabolites during fruit ripening will be useful for fruit improvement programs. Additionally, modeling techniques can predict the differences in fruit ripening stages **(9)**.

Therefore, considering the above considerations, this study aimed to investigate the antioxidant and antibacterial activities during fruit ripening in *V. corymbosum*.

2. Materials and Methods

Preparation of Microbial Suspension

Lyophilized bacterial ampoules were transferred to nutrient agar under sterile conditions and incubated at 37°C for 24 hours. The bacteria were then inoculated onto selective-differential agar and incubated at 37°C for 48 hours. A loopful of colonies was transferred to nutrient agar to prepare a fresh 24-hour culture to assess the antibacterial effects. Then, 1 mL of the 24-hour microbial suspension was transferred to sterile nutrient agar, and its turbidity was visually compared using a 0.5 McFarland standard prepared in the previous step. Nutrient agar and Mueller-Hinton agar were used for the tube dilution test and agar diffusion disk method, respectively, to determine growth inhibition and bactericidal concentrations **(10)**.

Evaluation of Antimicrobial Effects at Three Growth Stages

The antimicrobial effects of the fruit extracts were assessed using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests. To determine the MIC, 2-10 mg/mL were prepared in Mueller-Hinton broth. Each concentration was inoculated with a bacterial suspension equivalent to 1.5 × 10^8 CFU/mL. Nutrient agar without extract served as the positive control, and nutrient agar without bacteria served as the negative control. After incubation at 37°C for 24 hours, the microplates were examined for turbidity. The minimum concentration of the extract that showed no turbidity in the tubes was considered the minimum inhibitory concentration in mg/mL. For determining the MBC, 100 µL of the three tubes preceding the tube with the MIC concentration were linearly streaked onto Mueller-Hinton agar plates, and the minimum concentration of the extracts that showed no bacterial growth was reported as the MBC **(10)**.

Bacterial Killing Time Measurement

The killing time of *V. corymbosum* extract against *Salmonella* bacteria was measured according to the protocol provided by Appiah and Boakye **(10)**. The extract was used at MIC concentrations for the killing time measurement. Microplate wells were inoculated with 1.5 × 10^8 CFU/mL of each bacterial strain and incubated at 37°C. At 0, 0.5, 1, 2, 4, 6, 8, 18, and 24 hours, sufficient volumes of 1 mL from the culture medium were used for analysis. Extract-free and reference antibiotic-containing wells were used as negative and positive controls, respectively. The test was performed in triplicate, and log CFU/mL values were plotted against time.

Anticancer Properties

Hella (cervical cancer cells), MCF7 (breast cancer cells), and L929 (normal fibroblast cells) were obtained from the Pasteur Institute of Iran, Tehran. A humid atmosphere with 5% CO_2 was used for cell culture with DMEM supplemented with 10% FBS, streptomycin (1%), and penicillin (1%). The MTT assay was performed to evaluate cell viability. *V. corymbosum* extract at concentrations of 0, 0.5, 0.75, 1, and 1.5 mg/mL was used at three time points (24, 48, and 72 hours). In this study, cells were cultured at specific densities for each cell line. In summary, 10 µL of MTT was added to each well and incubated at 37°C for 4 hours, followed by the removal of the supernatant and the addition of 100 µL of DMSO. Absorbance at 590 nm was determined using an ELISA reader, and empty wells were used as controls. Additionally, the IC_{50} (50% inhibition concentration) was calculated based on the survival-log concentration curve. The MTT assay was performed with triplicates in all treatments **(11)**.

Statistical Analysis

Statistical analysis between groups was performed using Student's t-tests, ANOVA, and Origin Pro 2023 software for analysis ($P \leq 0.05$).

3. Results and Discussion

The results of the study involved determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of blueberry extract at different stages of fruit growth on the studied bacteria, specifically *Salmonella typhimurium*. The findings showed that unripe blueberry extract had a higher inhibitory effect against the tested bacteria. The MIC and MBC values for the unripe blueberry extract were determined as 3.5-3.8 mg/mL and 5.6-5.7 mg/mL, respectively. Additionally, semi-ripe and ripe fruit extracts also exhibited antimicrobial activity against bacteria at higher concentrations. The results indicate that *Salmonella* demonstrated greater sensitivity to blueberry extract during the early stages of fruit development.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of blueberry fruit extract against *S. typhimurium*.

	MIC(mg/mL)			MBC(mg/mL)		
	PG	BV	DB	PG	BV	DB
***Salmonella typhimurium*- Pathogen**	3.8	4.2	4.2	5.7	5.9	5.9
***Salmonella typhimurium* (ATCC14028) - Standard**	3.5	4.5	4.2	5.6	5.6	5.9

Time Killing of Blueberry Extract on *Salmonella* Growth in Three Developmental Stages

The findings from the time-killing study on Salmonella using blueberry extract in three developmental stages were examined. The impact of the killing time of *V. corymbosum* fruit extract on the growth of *S. typhimurium* at the MIC concentration for each extract was evaluated, and the results are presented in the provided figure. The results demonstrated that the killing time of the bacteria depended on the fruit's developmental stage, ripening, and duration of incubation. It was observed that after 18 hours of incubation, the unripe fruit extract led to complete inhibition of Salmonella growth (Figure 1). Furthermore, none of the fruit extracts showed any bacterial survival after 24 hours. The gradual reduction in the number of viable cells was observed during the initial 4, 6, and 8 hours when using MIC concentrations of the three extracts, followed by a more significant decrease. By the end of 24 hours, no bacterial growth was detected. The figure suggests that unripe fruit has a slightly better effect on reducing Salmonella growth, although the difference is not substantial.

The results revealed that blueberry extract had inhibitory effects against Salmonella bacteria, with the unripe fruit exhibiting a stronger inhibitory effect than other extracts (Table 1). Specifically, the unripe fruit showed the highest inhibitory activity, with a MIC of 3.5-3.8 mg/mL and an MBC of 5.6-5.7 mg/mL. Previous studies have also confirmed the antibacterial properties of berry extracts. Baenas and Ruales **(12)** identified blueberry extract as a natural antioxidant and anticancer agent, possessing in vitro and in vivo antioxidant capacity.

Their research demonstrated the sensitivity of S. aureus and E. coli to blueberry extract. Guevara-Terán, and Padilla-Arias **(13)** also highlighted the significance of harvest height and fruit ripening stages in inhibiting the growth of both Gram-positive and Gram-negative bacteria **(13)**. Phenolic compounds in fruit extracts have been suggested to influence bacterial cell growth. These compounds decrease the permeability of bacterial cell walls, resulting in disturbances in ion transport. Previous research on *V. floribundum* Kunth extracts demonstrated that high levels of phenolic compounds, particularly phenolic acids, organic acids, and flavonoid glycosides, hindered biofilm formation and the growth of bacterial species **(13)**. Studies on *V. macrocarpon* and *V. meridionale* evaluated the role of more complex phenolic polymers, such as proanthocyanidins. The antibacterial activity against both Gram-negative and Gram-positive bacteria was attributed to the presence of proanthocyanidins type A. These compounds increased bacterial membrane permeability, leading to pore formation, degradation, and subsequent cell death (14). Furthermore, Guevara-Terán and Padilla-Arias **(13)** found that polyphenols are the predominant compounds in berries, serving as the main bioactive compounds with antioxidant capacity and inhibitory effects against Gram-positive and Gram-negative bacteria. The effects were more pronounced in unripe fruit, which aligns with our findings. Previous reports have also indicated that the impact of blueberry extract on inhibiting bacterial growth varies depending on the developmental stage of the fruit. They also found that fruit extracts of *V. floribundum* at

different developmental stages and growth heights have varying inhibitory effects against Gram-positive bacteria like *E. faecium*, *E. faecalis*, and *S. aureus*, with MIC values ranging from 1.3-10 mg/mL **(13)**.

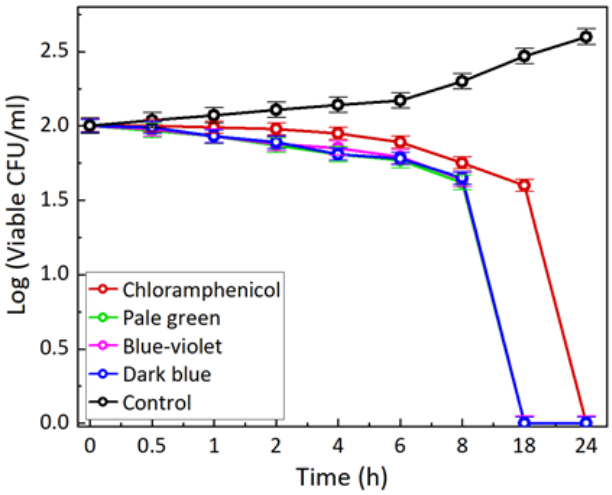

Figure 1. Time-killing curves of blueberry fruit extract (unripe fruit, semi-ripe fruit, and fully ripe fruit) against *Salmonella typhimurium*. The results are reported as the mean ± SE of three replicates ($P \leq 0.05$).

Anticancer Effect of Blueberry Extract on Cancer Cells at Three Stages of Growth.

The cytotoxic effect of *V. corymbosum* fruit extract on the growth of Hella and MCF7 cancer cell lines, as well as L929 normal cells, was evaluated using the MTT assay. The growth inhibitory effect of *V. corymbosum* fruit extract at three growth stages (unripe fruit, semi-ripe fruit, and fully ripe fruit) is depicted in Figure 2. Among the three extracts, the unripe fruit extract exhibited the highest cytotoxic activity against the studied cancer cell lines. At a concentration of 1 mg/mL and after 72 hours, it resulted in a cellular toxicity of 91.18% ± 1.65% for Hella cells and 90.05% ± 5.57% for MCF7 cells (Figure 2a, b). The cytotoxic effect of *V. corymbosum* fruit extract was more pronounced at higher concentrations, significantly impacting the cancer cell lines. After 72 hours of exposure to the extract at a concentration of 1 mg/mL, the cell viability in both cell lines was less than 10%. The cytotoxic effect of *V. corymbosum* fruit extract at the three growth stages on L929 normal cells was also evaluated. It was observed that the cell growth rate did not significantly decrease compared to the control group after 72 hours, with an increase in blueberry extract concentration to 1 mg/mL (Figure 2c). Moreover, the 50% inhibition of cell growth (IC_{50}) for all extracts in both cell lines was obtained between 0.70 mg/mL and 0.80 mg/mL after 24, 48, and 72 hours. The IC_{50} values indicated similar inhibitory effects on cell growth among the different extracts after different time intervals. Overall, the results showed that cell survival in the cancer cell lines depended not only on the concentration and exposure time but also on the growth stage of the fruit. The findings demonstrated a significant impact of the fruit's growth and developmental stage on cell growth, with the unripe fruit extract showing the highest inhibition of cell growth in all cancer cell lines after 72 hours at a concentration of 1 mg/mL.

Based on the results, it can be argued that the concentration of fruit extract, incubation time, and fruit ripening time are important factors in inhibiting the growth of cancer cells. Figure 2 illustrates that the minimum percentage of cell survival during incubation in both cell lines was observed with the fruit extract of the unripe variety at a concentration of 1.5 mg/mL after 72 hours, indicating that the unripe fruit extract had the most toxic effect on these two cell lines. The inhibitory effect of blueberry extract has also been confirmed in previous studies. It has been reported that blueberry, strawberry, and cranberry have strong anti-proliferative properties against human cervical cancer cell lines **(15)**. Various studies have shown that blueberry extract is an antiproliferative and apoptosis-inducing agent in colorectal cancer cell lines (HT-29 and HCT116) **(16)**. Hwang, Kim **(17)** reported that the anticancer activity of whole strawberry fruit is consistent between ripe and unripe Seolhyang varieties, while no significant difference was observed. However, unripe fruit showed more activity than ripe fruit in the Janghee variety. Yang, Kim **(18)** reported that unripe aronia fruit exhibits higher antioxidant activity than ripe fruit, which is consistent with the findings of this study. The antioxidant activity varies based on the variety and developmental stage of the fruit. Similarly, Shin, Ryu **(19)** found that immature strawberries of the Jewel variety have

higher antioxidant activity than fully ripe strawberries, aligning with the results of this research. Another study on blueberries showed that the concentrations of phenolic acids and flavonols were higher in unripe and fully ripe fruits, while the lowest levels were observed in semi-ripe fruits. This trend was also observed for total antioxidant activity, which could be attributed to the increase in phenolic acids during the immature stages of the fruit. Blueberries exhibited high overall antioxidant activity during ripening, with green fruit having the highest soluble phenolic content, and ripe fruit showing inhibitory activity against alpha-amylase and α-glucosidase compared to green or green/pink fruit (9).

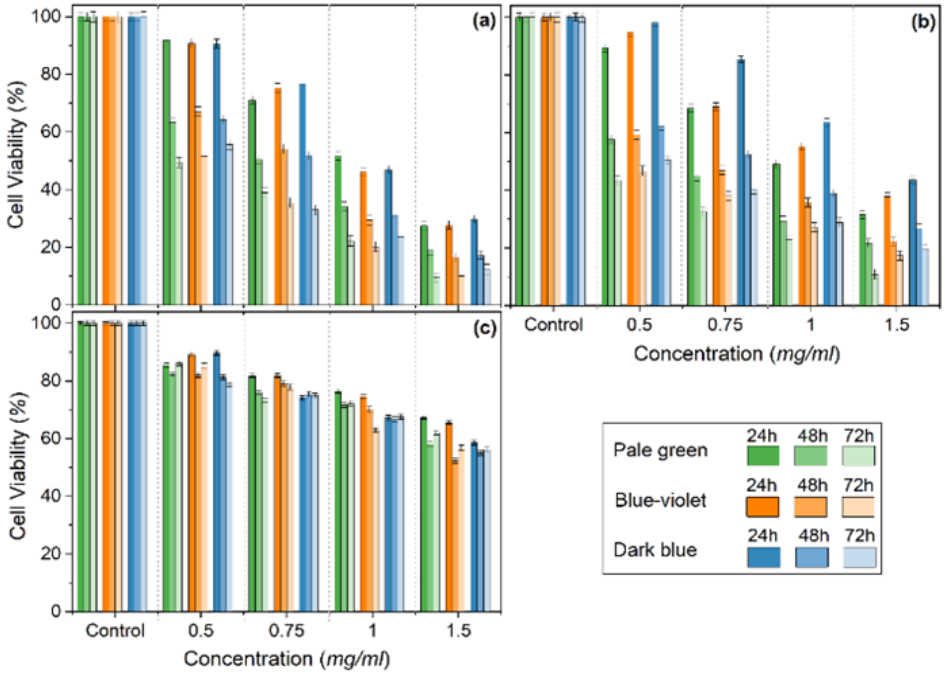

Figure 2. Effects of different concentrations of blueberry fruit extract at three growth stages: unripe fruit (pale green), semi-ripe fruit (blue-violet), and fully ripe fruit (dark blue) on (a) Hella, (b) MCF7, and (c) L929 cells after 24, 48, and 72 hours. Results are reported as the mean ± SE of three replicates ($P \leq 0.05$).

5. Conclusion

In conclusion, this study demonstrates that the antioxidant and antibacterial properties of blueberry extract are regulated throughout the ripening pattern of the fruit. Furthermore, the antibacterial properties of the unripe fruit extract were significantly better than those of the unripe and fully ripe fruit extracts. The inhibitory effects of the unripe fruit extract on cancer cell lines were also significant compared to other extracts. It can be concluded that the unripe fruit extract of blueberries may be a potential candidate for antibacterial and anticancer applications. However, further clinical trials are needed to validate the current findings.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' contribution

M.A.S: validation, formal analysis, investigation, writing-original draft preparation, writing review, and editing.

A.C.R: conceptualization, methodology, validation, investigation, writing-review and editing, funding acquisition.

S.A: methodology, validation, formal analysis, writing review and editing.

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