Vaccinium Vitis-Idaea L., Origin from Bulgaria Indicate in Vitro Antitumor Effect on Human Cervical and Breast Cancer Cells

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Abstract

Cancer is a socially significant disease. Along with efforts to understand the complex genetic/epigenetic factors that trigger a carcinogenesis, it is also necessary to analyze the potential natural active substances that may delay or even stop neoplastic transformation. Promising candidates are Bulgarian cranberries from high mountain plant populations, which are rich in phenolics and anthocyanins and have proven beneficial effects on human body. The present study aims to evaluate in vitro, antitumor activities of total extracts and purified nonanthocyanin and anthocyanins fractions of Vaccinium vitis-idaea L., picked in Bulgaria on human cervical (HeLa) and breast (MCF7) cancer cell lines, as well as to examine some of the apoptotic mechanisms underlying them.

Materials and methods: Four methanol extracts and respective number of purified B-nonanthocyanin / C- anthocyanins fractions of Bulgarian lingonberry were used. Antitumor effect was established by Trypan Blue method, monitoring of morphological changes and MTT cell viability assay. Assessment of apoptotic activity was performed using DNA fragmentation method.

Results: The results from MTT analyses showed that B- nonanthocyanin fractions of Bulgarian lingonberry have well expressed inhibitory effect on survival of tested tumor cells. The observed effect dependent of the dose administered and were stronger in relation with the high-mountain populations and HeLa cell line. The integrity of the extracted DNA from treated survival cells indicates possible apoptosis mechanisms under the action of biologically active ingredients from lingonberries.

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Conclusion: Survey of antitumor activities of Bulgarian lingonberries based on molecular methods, could contribute to establish the natural substances useful for human health in general and practical oncology.

**Keywords:** Vaccinium vitis-idaea L; antitumor activity; HeLa; MCF7.

1. Introduction

One alternative approach in cancer treatment is to analyze the antitumor activity of natural products known and used for therapy in traditional medicine. At present, several natural chemotherapeutic agents have been effectively used in oncology practice, such as paclitaxel *(Taxus brevifolia)*, vinblastine and vincristine *(Catharanthus roseus)*, artemisinin *(Artemisia annua)* [1]. For some of the active substances in plants, there is evidence that they induce apoptosis in different types of cancer cells. An example in this regard is Thaliblastine (an original Bulgarian medical product *Th. Aquilegifolium*) and Hyperatomarin (*H. annulatum*) with proven inhibitory effect on chronic myeloid leukemia cell lines [2]. Wild berries have been widely used based on its proven antiseptic properties, antioxidant and antimicrobial activities, since ages [3]. *Vaccinium vitis-idaea L.* (family *Ericaceae*, genus *Vaccinium*), also known as lingonberries refer to a group of functional foods. Nowadays a lot of drugs for the treatment of urogenital infections [4], cardiovascular and eyes disorders include berries as an active substants [5]. Lingonberries are rich in bioactive compounds such as anthocyanins, polyphenols, tannins, vitamins (A, C, E, B, PP etc.) and minerals [6]. A review of literature data shows that there is a strong interest and actively studies the antitumor potential of various active fractions of berries [7, 8]. To our knowledge, at present, no data exist concerning the antitumor activity of the Bulgarian lingonberries, except a single publication of Nikolaeva-Glomb and a team [9] evaluated antiviral potential of various wild berries, including lingonberries. In this regard the present study aims to investigate the in vitro antitumor potential of total extracts and purified fractions of Bulgarian lingonberries, through analysis of viability of human cervical (HeLa) and breast (MCF7) cancer cell lines after extract exposure, as well as to examine morphological changes of treated cancer cells underlying apoptosis progress.

2. Materials and methods

2.1. Plant Material

For the purposes of the analyses four plant populations of wild lingonberries growing at different altitudes in the regions of mountains - Balkan (Stara Planina) and Rhodope were selected. The ripe berries were collected at the time period when they are typically harvested for commercial purposes. Bushes were randomly selected within the populations, on the precondition that the minimum distance between the studied plants was 10 m. The previous data show variation in the chemical composition and content of biologically active substances in the *Vaccinium vitis-idaea L.* inhabiting various geographic regions and growing under specific soil conditions [10]. In connection with this approach fruit from wild populations Vaccinium vitis-idaea L. with habitat in the Balkan (Vasiliov - GPS: 42°52.753’N 24°28.968’E; altitude: 1360 m and Beclemeto - GPS: 42 46.460’N 24 37.000’E altitude: 1470 m) and the Rhodope Mountains (Perelik - GPS: 41°36.400’N 24°35.805’E; altitude: 1970 m and Gela - GPS: 41°37.967’N 24°33.183’E altitude: 1780 m) in Bulgaria were collected in 2018. All samples were freeze-dried, ground and stored at – 80°C prior to extraction.
2.2. Plant Extraction

Then extraction solution consisted of 80% solvent A [AcN-MeOH (80:20 v/v)] and 20% solvent B [0.1 % aqueous HCOOH] were added. The samples were sonicated (10 min), and centrifuged (12000 rpm, 5 min, 4°C). The filter cake was reextracted two times with extraction solution. The supernatants were combined and dried in vacuum-concentrator. The extracts were fractionated by Solid Phase Extraction (SPE) using a Giga tubes 2 g/12 ml, C18-E units (Strata, Phenomenex®). The columns were activated with 0.1% (v/v) formic acid in AcN then followed by EtOAc and 0.1% (v/v) HCOOH in water. Water-soluble compounds (fraction ‘A’) - sugars, organic and amino acids were removed with 2 volumes (2 x 12 ml) 0.1% (v/v) formic acid in water. The non-anthocyanin components (phenolic acids, flavonols, and condensed tannins -fractions ‘B’) were eluted from the columns with 2 volumes (2 x 12 ml) EtOAc. Finally the anthocyanins (fractions ‘C’) were be subsequently eluted with 2 volumes (2 x 12 ml) 0.1% (v/v) HCOOH in AcN. Fractions ‘A’ were not retained. Fractions ‘B’ and ‘C’ were dried using a vacuum-concentrator.

2.3. Sample descriptions

Tv, Tb, Tp and Tg – total extracts from Vaccinium vitis-idaea L, picked in the regions of mountains: Balkan - locations Vasiliov (Tv) and Beclemeto (Tb) and Rhodope - locations Perelik (Tp) and Gela (Tg) respectively. Cv, Cb, Cp and Cg - the -anthocyanin and Bv, Bb, Bp and Bg the – non – anthocyanin (polyphenol) fruits fractions from lingonberries corresponding to the total extracts from the above described habitats.

2.4. Cell lines and cultivation

Human Breast (MCF7) and cervical (HeLa) cancer cell lines were included in the study. HeLa cell line is obtained from a patient with cervical cancer. The line has been widely used for in vitro analyzes for over fifty years. MCF7 is isolated from pleural effusion of a 69-year old Caucasian woman with metastatic breast cancer. The cell lines were supplied by the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% sodium pyruvate (Eurobio) and 1% MEM Non-Essential Amino Acids (Gibco) and were incubated at 37°C in a humidified atmosphere with 5% CO2. The cell lines were kept free from fungal or bacterial contamination.

2.5. Trypan blue exclusion assay

Trypan blue exclusion assay was carried out to calculate percentage of cell lines MCF7/HeLa viability. An aliquot of 50μl cell suspension was mixed with an equal volume of 0.4% trypan blue solution. Viable (unstained) and nonviable (dark blue stained) cells were counted under inverted light microscope. Percent viability was calculated by the formula: Viability (%) = (Live cell count/Total cell count) x 100

2.6. MTT assay

In vitro cell viability was evaluated through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium
bromide) assay. Cells were seeded into 96-well tissue culture plates (1x10^5 per well) in a final volume of 100 µl. After incubation for 24 h in complete cell culture medium, for the next 24 h cells were starved in serum-free medium, supplemented with 0.1% BSA. Subsequently cells were treated with different concentrations of tested extract (from 0.5 to 1000 µg berry/ml medium) for 48h/72h using cultivating medium as a solvent. Wells with serum-free medium were used as negative controls. During the last 3 h of the incubation an aliquot of 10 µl MTT per well was added (stock solution of 5 mg/ml MTT was used). After incubation, the medium was removed and the formazan complex was dissolved with 100 µl/well 10% SDS in 0.01M HCl. The absorbance was subsequently measured at 570 nm using ELISA microplate reader. The MTT test was repeated at least twice and each concentration had three repeats. The percentage of cell viability after extract exposure of the above-mentioned concentrations was determined using the following formula: Viability (%) = (Experiment value/Control value) \times 100, and the 50% inhibitory concentration (IC50) was determined. MTT analysis was also applied to establishment of cell viability alteration with time after treatment - for 48 and 72 h.

2.7. Morphological observation of tumor cells

MCF7 and HeLa cells were plated into 12-well plates, cultivated and treated for 48 and 72 h with the same concentrations, as described above. Morphological changes were observed using inverted light microscope. The morphological observation analysis was synchronized with MTT cell viability assay.

2.8. DNA fragmentation analysis

Cells were plated into 6-well culture plates (1x10^5 per well). After treatment with total extract, fractions ‘C’ and ‘B’ from lingonberries for 24h, 48 h and 72 h the cells were detached with 0.05% trypsin-0.53 mM EDTA, incubated for 15 min at 37°C and collected. After centrifugation for 5 min at 125G the supernatant was discarded and 200µl PBS pH 7.2 was added to the cell pellet. DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen). An aliquot of 2.5 µg genomic DNA was analysed on 1.5% agarose gel in 1xTBE, at 80 V and 50 mA for 1 h. The samples were visualized under UV after gel staining with ethidium bromide.

3. Results

3.1. Cell Viability

Using Trypan blue exclusion test percentage cell lines viability was assessed. The results showed that the percentage viability of MCF7 cell line was 96.03% ± 2.47(SD) and HeLa was 95.02% ± 2.71(SD), which is suitable to perform the following tests. The antitumor effect of lingonberries total extracts - Tv, Tb, Tp and Tg, fractions ‘C’ - Cv, Cb, Cp and Cg and ‘B’ - Bv, Bb, Bp and Bg were examined through MTT cell viability assay. Untreated tumor cells were used as vehicle controls and their viability was accepted as 100%. The screening was performed at multiple concentrations ranging from 5 to 1000 µg/ml. The range of concentrations (5, 10, 20, 32, 100, 320, 1000 µg B/ml) for MTT was selected according to previous data for nontoxic properties and an effect (antiviral) of total extract and/or fractions from wild berries on viability of human cell lines [9].

- MTT assay on total methanol extracts and C – anthocyanin fruits fractions of *Vaccinium vitis-idaea L.*
picked in Bulgaria on human breast and cervical cancer cell lines.

The results showed lack of effect on treated tumor cells and a high survival rate (100% and over) (increased compared to untreated controls) in treatment for both 48 and 72 hours at all concentrations tested (5, 10, 20, 32, 100, 320, 1000 µg Berry/ml media) and for all four total extracts ((Tv, Tb, Tp and Tg) and also for C – anthocyanin fruits fractions of lingonberry picked in Bulgaria on the cells from MCF7 and HeLa lines.

- MTT assay on B non – anthocyanin fruits fractions of lingonberry on MCF7 and HeLa cell lines.

The here obtained data indicated that B – non anthocyanins fractions from lingonberries picked in Bulgaria had a marked dose and time dependent effect on viability of breast and cervical cell lines. In the range of lower concentrations (up to 5 µg B/ml medium) the viability of tumor cells is comparable to this in the control and with the increase of concentration a steady decrease in cancer cells viability was observed (Figure 1). Fifty percent (IC50) reduction of tumor cell viability was observed at a concentrations rate 100 - 320µg B /ml medium for HeLa cells and 320- 1000µg B /ml medium for MCF7 cells treated for 48 and 72 hours with B - phenolic fractions from the representatives of different populations raised in Bulgaria. The most significant anti-tumor effect was determined in B fractions from lingonberries from higher mountains populations.

![Figure 1](image_url)

**Figure 1:** MTT assay of HeLa (A/B) and MCF7 (C/D) cell lines treated for 48 h (A/C) and 72 h (B/D) with increasing range of concentrations of B – non anthocyanins fractions from Vaccinium Vitis-idaea L picked in the regions of mountains: Balkan - locations Vasiliov (Bv) and Beclemeto (Bb) and Rhodope - locations Perelik (Bp) and Gela (Bg).
The statistical analysis according to GraphPad Prism indicated considerable significant differences between control and treated groups with p-values of less than 0.05.

3.2. DNA fragmentation analysis

Cells from HeLa and MCF7 lines were treated with 100 μg/ml of B non – anthocyanin fruits fractions of *Vaccinium Vitis-idaea L.* for 24 h, 48 h and 72 h. The results showed presence of DNA fragmentation both in breast and cervical cancer cells after 24 h treatment with B non – anthocyanin fruits fractions. Slight increase in the level of DNA fragmentation in both cancer cell lines with extension of duration treatment (48 h and 72 h) was indicated.

3.3. Morphological Alterations

Morphological changes of treated MCF7 and HeLa cancer cells were observed under inverted light microscope. Extract treated cells became round and shrank in comparison with untreated control cells with normal shape. Evident reduction of viable cells which were monolayer adherent and increased number of floating dead cells after treatment with concentrations above 320 µg/ml were observed. The number of tumor cells detached from the monolayer increase in extract concentration.

4. Discussion

At present plant products are intensively studied as a potential source of active components for cancer therapy. From 1940 to 2010, 48.6% of the 175 small molecules approved in oncology treatment were natural- or natural derived products [11]. The biological activity of the medical plants depends of the chemical composition. Phytochemicals, especially phenolic in berries, are suggested to be the major bioactive compounds responsible for their health benefits. Phenolic extracts of plant materials are always a mixture of different classes such compounds that are soluble in the solvent system used [12, 13]. Vaccinium berries have been shown to contain high levels of the flavanoid compounds that contains a common molecular structure - tricyclic C6-C3-C6 “flavanoid skeleton” [14, 15]. The high phenolic rate in *Vaccinium vitis-idaea L.* and the data on their antimutagenic, anti-inflammatory and antimicrobial activities presumes a high antitumor potential of this medicinal plant [16, 6].To our knowledge, the present study reported the first data on the antitumor effect of Bulgarian *Vaccinium vitis-idaea L.* The investigations were carried out on breast adenocarcinoma MCF7 and HeLa cervical cancer cell lines through MTT assay for cell viability in a wide range of extract concentrations. A profound reduction in tumor cell viability was found after treatment with B non – anthocyanin fractions. The effect was dose and time dependent resulting in decrease in cell viability of treated cancer cells. The non – anthocyanin fraction had stronger inhibitory effect on viability of cervical compared to the breast cancer cells. Here we found that IC50 of B non – anthocyanin fruits fractions from different plant populations varies in the range of concentrations for HeLa and MCF7 cells treated for 48 and 72 hours and were lowest for lingonberries growing at high altitudes. By the moment data from a scientific reports represent that berry fruits such as blueberries, strawberries, raspberries and lingonberries, inhibits the growth of human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT116), prostate (LNCaP) tumor cell lines and may influence on multiple stages of
In accordance with our results other authors also detected increasing inhibition of cell proliferation rate after treatment with increasing concentration of berry extract in a wide spectrum of cell lines tested [20, 7, 21-24, 8]. The variations in IC50 values between publications are probably due both to the different drug sensitivity of studied tumor cell lines, diversity in chemical composition and environmental conditions of plants from different geographical areas and populations. Higher amounts of total phenolics were detected in samples harvested from localities exposed to the sun in comparison with berries grown in shadow. It has been noticed that at altitude higher than 1500 m, higher amounts of total phenolics in the lingonberry was observed [15]. Some phytochemicals, contained in fruits of the Vaccinium genus, are expected to affect cancer-related processes. Phenolics, proanthocyanidins and flavonoids, presented in lingonberries and other Vaccinium berries, show some promising effects toward limiting processes involved in tumor invasion, apoptosis and metastasis [8, 25, 26, 19]. Apoptosis (programmed cell death) is initiated in response to damages in hereditary material and represents a series of genetically controlled events, resulting in elimination of damaged cells. The process is associated with activation of cellular endonucleases, which digest cellular DNA to well-differentiated fragments that can be visualized through gel electrophoresis [27]. Our results showed induction of apoptosis after treatment with B non – anthocyanin fruits fractions of Vaccinium vitis-idaea L. both in breast and cervical cancer cells. This observation is in accordance with the data from the MTT assay and by increasing the duration of treatment with polyphenol fractions, DNA fragmentation level in tumor cells slightly increased. This is consistent with the observations that DNA fragmentation occurs in the later stages of the apoptosis [28]. Similar data for induction of apoptosis are also obtained in the analysis of the effect of V. oxyccocos fruits on breast cancer cells. Masoudi M and Saiedi M [17] found that V. oxyccocos fruits were able to suppress the proliferation of human MCF7 cells and attributed to the initiation of apoptosis and the G1 phase arrest. In conclusion, B non – anthocyanin fruits fractions of Bulgarian Vaccinium vitis-idaea L. has a marked dose-dependent inhibitory effect on viability of breast and cervical cancer cells. In the mechanisms underlying the antitumor effect of Vaccinium vitis-idaea L., apoptotic processes are involved. Morphological changes and DNA fragmentation were observed as markers for apoptosis process in tumor cells after treatment. The obtained results are the first showing an antitumor activity of the Bulgarian Vaccinium vitis-idaea L. in human cancer cells and are indicative for further more detailed investigations concerning detailed screening for the active compounds determining the antitumor activity of the plant.

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