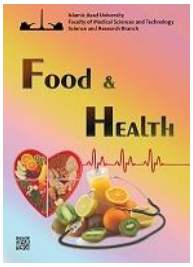

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# Investigating antitumorigenic effects of *Vaccinium arctostaphylos* on colorectal cancer cells

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ABSTRACT

Anticancer and anti-inflammatory effects of American and European Cranberries have been previously shown on different cancer cells. Owing to the limited evidence on growth conditions and anticancer potentials of the Iranian Vaccinium genus, *Vaccinium arctostaphylos*, it was aimed to investigate its effect on colorectal cancer cells. In this regard, Vaccinium *arctostaphylos* was cultured in Woody Plant Medium (WPM) following incubation at 27°C in cycles of light and darkness. Callogenesis was induced using growth mediums containing different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D), yeast extract, and Kinetin. Total polyphenol and antioxidant activity of prepared extracts from wet and dried callus and air-dried fruit was measured through the Folin-Ciocalteu method and DPPH assay, respectively. Various concentrations (0-500 µgr/ml) of fruit and callus extracts were examined on HCT-116 colorectal cancer cells. MTT assay was employed to determine the cytotoxicity of fruit and callus extracts. Obtained data were analyzed using Graph Pad Prism V7.04. The size and weight of the obtained callus were significantly dependent on the concentrations of 2,4-D, yeast extract, and Kinetin. Dry callus has been found to have the highest amount of polyphenol and antioxidant activity. HCT-116 cell death rate (20.5%) was demonstrated to be the most for dry callus at the concentration of 400 µg/ml. However, half-maximal inhibitory concentration ($IC_{50}$) was not achieved for any of *Vaccinium arctostaphylos* fruit or callus, Present evidence on cancer cell death can pave the way towards further assessment of anti-inflammatory and cancer cell cytotoxicity of the Iranian Cranberry genus.



## 1. Introduction

Colorectal cancer (CRC) is one of the most frequent human cancers in the population, worldwide. CRC is the second leading cause of cancer-related mortality and despite current screening diagnostic tests and colonoscopy, more than one-third of the patients were identified in advanced stages (1). Although adjuvant chemotherapy has improved the Overall survival (OS) of CRC patients, it is still low in most metastatic cases (2). Targeted therapy has revealed promising horizons in increasing the OS of CRC patients. However, it is not cost-benefit, and in addition to having multiple severe side effects, targeting some pathways may lead to drug resistance, exacerbating the therapy drawbacks. In addition, it was found that targeted therapy may be helpful in specific patients and, therefore, requires pretreatment screening (3). It was recently demonstrated that dietary factors play pivotal roles in CRC development and fruits are among the factors with significant revere association (4). Recent investigations were focused on the therapeutic role of dietary factors in CRC by increasing the OS of cancer patients. Different parts of the diet have been extensively investigated for the prevention and treatment of CRC, including dietary fiber, omega-3 fatty acids, vitamin D, minerals, and calcium intake. Although there are still controversies among different studies on the association between helpful dietary factors and improving OS, bioactive dietary compounds, as well as polyphenols, have seized great attention in late research (5, 6). It was demonstrated that polyphenols have great anti-inflammatory and immunomodulatory effects in favor of cancer cell regression.

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Polyphenols modulate immunoreaction through suppression of T regulatory cells and some of the key proinflammatory cytokines including IL-6, IL-1β, TNF-α, and IL-12 suppression and, co-stimulation of T cells in CRC cells. Moreover, polyphenols act as prebiotics and, therefore, have critical roles in maintaining the gut microbiota and improvement in colon health through exciting the growth of helpful bacteria. It was recently reviewed that modulating the gut microbiota can enhance the anti-tumor effects of chemotherapeutic drugs and immune checkpoint inhibitors (7, 8). Interestingly, polyphenols are the potential drugs for targeting epigenetic alteration made during cancer evolution. They can cause global DNA hypomethylation through the decrease in S-adenosylmethionine (SAM) and suppressing or impeding DNA methyltransferase (DNMT) function (7). Polyphenols can reverse miRNA-based cancer epigenetics changes by direct inhibition of miRNA maturation and thereby suppressing oncogenic miRNA as well as miR-27a or increasing the expression of tumor suppressor miRNA and miR-663 (8). Polyphenols can be categorized into two main classes Flavonoids and non-Flavonoids. Flavonoid subclasses include anthocyanidins, flavanols, isoflavones, flavanones, and flavanones. Cranberry or *Vaccinium macrocarpon* Ait. Ericaceae is one of the fruits which is rich in flavan-3-ols, anthocyanins, and flavanols and has more polyphenols in comparison with other types of Berries or apples. It is mainly cultured in North America (especially in Massachusetts) and is well known for its amazing therapeutic and preventive effects on urinary tract infections. Although different bioactive components of cranberry extract can exert anticancer effects, their interaction with various cancer cell lines has been demonstrated to be different. In the treatment of different berries on two PC-3 and MDA-MB-231 cancer cells, cranberry juice and extract have demonstrated the most toxicity and apoptosis induction through suppression of chief cyclin-dependent kinases 4/6 and cyclin D1/D3 (9). Polyphenolic fractions of cranberry induced anti-inflammatory effects on Caco-2/15 cells through downregulation of nuclear factor κB and thereby decreasing oxidative stress (10). Treatment of prostate cancer cells with ursolic acid and esters derived from Vaccinium macrocarpon as the American cranberry demonstrated downregulation of MMP-2 and MMP-9 and therefore, tumor invasion (11). Flavonoid and proanthocyanidin fractions derived from fresh and press cake the American cranberry showed inhibitory effects on colon carcinoma cells (HT29) explant proliferation as well as glioblastoma multiforme and prostate cancer cells (12). Dang Vu et al. also tested the anti-proliferative effects of flavonoid and anthocyanins fractions derived from different forms of *Vaccinium macrocarpon*, including frozen, puree, depectinised puree and pomace on HT-29 and LS-513 CRC cells. Although, all the types of extracts were effective on limiting the growth of both cell lines, the anticancer efficiency of pomace was found to be the least (13). Asgari et la, have shown insignificant anti-inflammatory effect of *Vaccinium arctostaphylos*, in Western Asia (Iran and Turkey) species of cranberry extract in the serum inflammatory cytokines markers (14). To the best of our knowledge, the anticancer effect of *Vaccinium arctostaphylos* was only examined on prostate cancer cells which were associated with significant upregulation of *GSTP1* gene (15). Herein, it was aimed to assess the anticancer effects of *Vaccinium arctostaphylos* on colorectal cancer cell models in the different form of dried, fresh, and frozen forms.

## 2. Materials and methods

*Vaccinium arctostaphylos* seeds were purchased from X company and then were cultivated in glass Petri dishes containing sterilized distilled water in the incubator at 27C and a light cycle of 16 hours of lightness and 8 hours of darkness. Following appearing the cotyledons of the plant, it was transferred to 1/3 Murashige and Skoog (MS) plant culture medium and then Woody Plant Medium (WPM) including 1mg/ml Zeatin plant growth hormone (GoldBio-USA) for stem formation. Callogenesis was induced through further culture of *Vaccinium arctostaphylos* in basic WPM and MS mediums including 2,4-Dichlorophenoxyacetic acid (2,4-D, 1 and 3 mg/ml), (0.1 and 0.5 mg/ml) and yeast Extract (0.25 % and 0.5 %). Culturing of the *Vaccinium arctostaphylos* was performed in 28 replicates and followed up for 8 weeks in the darkness. Callogenesis rate, the calluses' size and weight of wet and dried calluses were determined during the callogenesis phase.

### *2.1. Extract preparation from semi-dried fruit and callus*

Two grams of each wet and dried callus in addition to the dried fruits were grounded in liquid nitrogen using pestle and mortar and, then resuspended in 100 ml of ethanol 70% and incubated at 60C° for 20 minutes. For efficient cell wall degradation and cellular blebbing, the plant solution was sonicated at 40 kHz for 20 minutes using Cleaner Ultrasonic WUC-A03H Liter (Australia). After an overnight shaking, the plant lysate in the darkness, it was filtered through filter paper (2µm).

### *2.2. Measurement of total polyphenol*

Different concentrations of Gallic acid including 0, 10, 25, 50, 75, and 100 mg/ml were used as standard concentrations to draw standard curves. Five ml of Folin-ciocalteu reagent (10%) was mixed with 1mgr of each Gallic acid concentration and every plant extract. 1.5 ml of $Na_2CO_3$ solution (20%) was then added to the previous mixture and adjusted to the final volume of 10 ml using $ddH_2O$. Following incubation in the darkness for 90 minutes, all the solutions were analyzed at 765nm via spectrophotometry (16).

### *2.3. Antioxidant characteristics evaluation*

Evaluation of antioxidant characteristics of the plant was performed through DPPH assay. Four different concentrations were prepared from each wet, dried fruit, and dried callus and

then mixed with the same volume of DPPH ($6\times10^{-5}$ M, dissolved in ethanol 70%). After incubation at room temperature and darkness for 30 minutes, solutions along with their blank control containing DPPH and 70% of ethanol (1/1) were measured at 520 nm via spectrophotometry.

### *2.4. Cell culture*

To investigate the anticancer potentials of antioxidant and polyphenol of cranberry callus and fruit, they were treated on HCT-116 colorectal cancer cell line. In this regard, HCT-116 cells were cultured in DMEM medium supplemented with FBS (5%) and pen/strep (1%) and then treated with different concentrations of 0-500 µgr/ml of fruit and callus extracts resuspended in FBS (1mgr/ml).

### *2.5. MTT assay*

MTT assay kit (Sigma, St. Louis, MO) was used to measure the inhibitory effect of fruit and callus extracts on HCT-116 cell growth. A total of $10^4$ cells were seeded in each well containing DMEM medium, 10 % FBS, and 1% penicillin/streptomycin. After 24 hours, cells were treated with fruit and callus extracts with different concentrations of 0, 100, 150, 200, 250, 300, 350, 400, 450, and 500 mg/ml in triplicates and each plate was repeated in two other separated experiments. Plates were incubated for 24 and 48 and 72 hours at 37˚C under 5% (v/v) $CO_2$. Cell viability was measured by ELISA reader (Anthons2020, version 1.8.3, UK) and analysis was performed using the following equation.

$$\%\text{Cell viability} = \frac{Absorbance\ of\ sample - Absorbance\ of\ blank}{Absorbance\ of\ Control - Absorbance\ of\ blank} \times 100$$

### *2.6. Statistical analysis*

Graph Pad Prism V7.04 has been used to perform all statistics in this study. The half-maximal inhibitory concentration ($IC_{50}$) was calculated, using non-linear regression. Normality distribution and variance Homogeneity between groups were tested, using the Shapiro Wilk test and Brown Forsythe test respectively. To compare between groups, a regular one-way or two-way ANOVA test has been used based on the dimensions of the data. $p<0.05$ was assumed as a significant threshold.

## 3. **Results**

### *3.1. Callogenesis*

Callogenesis was performed in different WPM mediums including 1 and 3 mg of 2,4-D hormone, 0, 0.1, and 0.5 mg/ml concentrations of Kin and 0, 25% and 50% of yeast Extract. Variance analysis on the mean of callogenesis percent was not significantly different among the 14 experiments (p-value=0.134). However, the size of obtained calluses was significantly larger in medium, containing 1 mg/ml of 2,4-D, 0.5 mg/l of Kin and 5% of yeast extract (p-value<0.0001) in Duncan analysis. Maximum wet weight of callus was obtained in medium with 1 mg/ml 2,4-D, 0.5 mg/l of kin and 0.5% of yeast extract (p-value<0.0001). However, the maximum of dried weight of callus was achieved in a medium with 1 mg/ml 2,4-D, 0.1 mg/l of kin, and 0.5% of yeast extract (p-value=0.004).

### *3.2. Measurement of polyphenols*

The polyphenols of wet and Dried callus and the dried fruits were measured through the Folin-Ciocalteu method (16). Dried callus extract had the highest quantity of polyphenol (473.71 mg GAE/ 100 ml) in comparison with maximum polyphenols of 0.82 and 0.37 in dried fruits and wet callus, respectively.

### *3.3. Antioxidant activity assay*

The absorbance rate of free radicals was measured through the DDPH method in all the samples and the results were presented in Table 1.

**Table 1**. Absorbance rate of free radicals in different concentrations of Dry and wet callus and dried fruit extracts.

| Concentration | Dry Callus | Wet Callus | Dried fruit |
|---|---|---|---|
| %200 | 72.72 | 54.4 | 53.44 |
| %100 | 61.36 | 42.06 | 38.83 |
| %50 | 49.54 | 33.17 | 34.23 |
| %25 | 34.77 | 27.16 | 23.38 |

### *3.4. MTT assay*

It was demonstrated that cancer cell death was enhanced with an increase in the concentration of dried fruit extract which was maximum (19%) at a concentration of 500 µg/ml (Fig. 1). However, cancer cell death was observed in concentrations of more than 300 µg/ml of dry callus which was maximum at 400 µg/ml (20.5%).

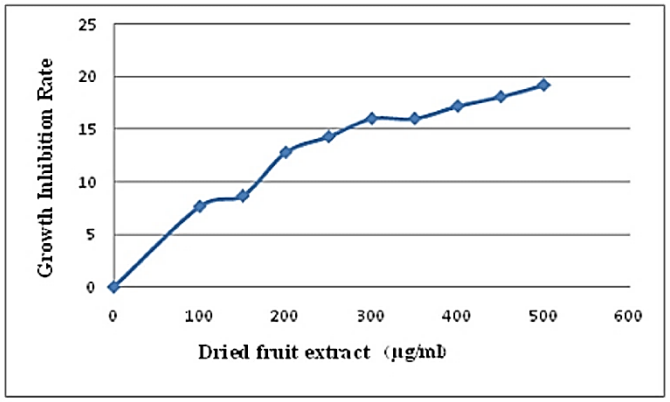


**Fig. 1**. Growth inhibitory effect of the different concentrations of dried *Vaccinium arctostaphylos* fruit extract on HCT-116 cells.

## 4. **Discussion**

Harvesting cranberries in WPM medium demonstrates that

interaction between kin and 2,4-D had a critical effect on its callogenesis. It was shown that the concentrations of calluses were significantly higher in medium containing kin compared to medium devoid of it. Similarly, Karimian et al. (17) have found that culture medium containing 1.5 mg/L of 2, 4-dichlorophenoxyacetic acid and 0.1 mg/L of Kinetin was the most suitable combination for callogenesis of Taxus Brevifolia Nutt. Essential role of Kinetin on callogenesis of *V. corymbosum* L. cv. Sunt Blue Giant was confirmed, as well. Moreover, Rashmi et al. demonstrated that 2,4-D containing medium was the strongest one in callus induction (18). To the best of our knowledge, this is the first trial of using a combination of yeast extract, kin and 2,4-D in a culture medium of *Vaccinium arctostaphylos*. Recently, Farjaminezhad and Garoosi (19) have shown that supplementation of plant culture medium with yeast extract can dramatically increase the production of secondary metabolites of Azadirachta indica cells and their growth. In the present work, Maximum polyphenol was found in dried callus which was more than in dried fruit. Dried fruits are commonly not propagated and are exposed to weather changes which can dramatically affect polyphenolic concentration (20). Free radical scavenging was found to be meaningfully increased in 200% concentrations of Each analyzed extract which were the maximum in dried callus. Similarly, Ziemlewska et al. (21) demonstrated more antioxidant activity in higher concentrations of three types of berry leaf extracts. It was also shown that dried cranberry has more Chlorogenic Acid with substantial anticarcinogenic and chemopreventive activity (22). There are several studies that indicate high antioxidants and polyphenols concentrations in different types of fruits, in line with our findings Regarding Cranberry (23, 24). Although the half-maximal inhibitory concentration ($IC_{50}$) was not obtained with any of the concentrations of the extracts, decreasing cancer cell proliferation can rely on the anticancer effects of *Vaccinium macrocarpon.* Interestingly, this insignificant Anti-proliferative effect was observed despite the higher polyphenol concentration of the Iranian Vaccinium macrocarpon compared to other species. Tunde Jurikova et al. (25) demonstrated that the European *Vaccinium macrocarpon* has 12.4–207.3 mg/100 g fw polyphenolic compound which is considerably lower than the dried callus of our examined *Vaccinium arctostaphylos*. Interestingly, obtaining polyphenol in the Asian Vaccinium is even more than reported American species as well as *V. macrocarpon* Aiton, which is well known as large cranberry (5.34 ±0.026 mg RE/g DW). It was found that it is rich in anthocyanins, phenolic acids, flavan-3-ols, and flavones and, therefore, has sized great attentions to be grown in other countries. (26). Either American and European cranberry species have demonstrated significant cytotoxicity against colorectal cancer cells (27, 28). However, despite alleviating the proliferation rate of Caco-2 cells, cell death was not reached to 50% for all of the extracts. Recently, Yu et al. (29), demonstrated that $IC_{50}$ is not a satisfactory outcome of anticancer effects of drugs due to its vulnerability to being influenced by cellular doubling time. In addition, colorimetric cytotoxicity assays like MTT can have high false positive and false negative results in testing foods containing polyphenols as well as Cranberry (30). Moreover, cancer preventive foods are not necessarily anticancer, as well and, most cancer-preventive foods should be limited in the diet of cancer patients, in particular, metastatic ones (30). In this regard, further studies are required to shed light on the anticancer and cancer cell growth inhibitory effects of cranberry on colorectal cancer cells through gene and protein analysis.

## 5. Conclusion

Despite reaching no $IC_{50}$, *Vaccinium arctostaphylos* as Western Asian species of Cranberry reduced colorectal cancer cells in the present study. Owing to the frequent use of *Vaccinium arctostaphylos* in Iranian therapeutic diets, Further studies are strongly recommended to elucidate its role in cancer cell proliferation and metastasis.

## References


1. Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, et al. Cancer treatment and survivorship statistics, 2016. *CA: A Cancer Journal for Clinicians*. 2016;66(4):271-89.
2. Yee NS. Update in systemic and targeted therapies in gastrointestinal oncology. *Biomedicines*. 2018;6(1):34.
3. Xie Y-H, Chen Y-X, Fang J-Y. Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduction and Targeted Therapy*. 2020;5(1):22.
4. Papadimitriou N, Bouras E, van den Brandt PA, Muller DC, Papadopoulou A, Heath AK, et al. A prospective diet-wide association study for risk of colorectal cancer in EPIC. *Clinical Gastroenterology and Hepatology*. 2022;20(4):864-873.e13.
5. Thanikachalam K, Khan G. Colorectal cancer and nutrition. *Nutrients*. 2019;11(1):164
6. Van Blarigan EL, Zhang S, Ou FS, Venlo A, Ng K, Atreya C, et al. Association of diet quality with survival among people with metastatic colorectal cancer in the cancer and leukemia b and southwest oncology group 80405 trial. *JAMA Network Open*. 2020;3(10):e2023500.
7. Arora I, Sharma M, Tollefsbol TO. Combinatorial epigenetics impact of polyphenols and phytochemicals in cancer prevention and therapy. *International Journal of Molecular Sciences*. 2019;20(18):4567.
8. Biersack B. Current state of phenolic and terpenoidal dietary factors and natural products as non-coding RNA/microRNA modulators for improved cancer therapy and prevention. *Non-coding RNA Research*. 2016;1(1):12-34.
9. Boivin D, Blanchette M, Barrette S, Moghrabi A, Béliveau R. Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NFkappaB by edible berry juice. *Anticancer Research*. 2007;27(2):937-48.
10. Denis MC, Desjardins Y, Furtos A, Marcil V, Dudonné S, Montoudis A, et al. Prevention of oxidative stress, inflammation and mitochondrial dysfunction in the intestine by different cranberry phenolic fractions. *Clinical Science*. 2015;128(3):197-212.
11. Kondo M, MacKinnon SL, Craft CC, Matchett MD, Hurta RA, Neto CC. Ursolic acid and its esters: occurrence in cranberries and other Vaccinium fruit and effects on matrix metalloproteinase activity in DU145 prostate tumor cells. *Journal of the Science of Food and Agriculture*. 2021;30;91(5):789-96.
12. Ferguson PJ, Kurowska EM, Freeman DJ, Chambers AF, Koropatnick J. In vivo inhibition of growth of human tumor lines by flavonoid fractions from cranberry extract. *Nutrition and Cancer*. 2006;56(1):86-94.
13. Vu KD, Carlettini H, Bouvet J, Côté J, Doyon G, Sylvain J-F, et al. Effect of different cranberry extracts and juices during cranberry juice processing on the antiproliferative activity against two colon cancer cell lines. *Food Chemistry*. 2012;132(2):959-67.
14. Asgary S, Soltani R, Mirvakili S, Sarrafzadegan N. Evaluation of the effect of Vaccinium arctostaphylos L. fruit extract on serum inflammatory

biomarkers in adult hyperlipidemic patients: a randomized double-blind placebo-controlled clinical trial. *Research in Pharmaceutical Sciences*. 2016;11(4):343-8.
15. Gorbanzadeh E, Zaefizadeh M. Inhibitory effects of vaccinium arctostaphylos extract on prostate cancer cells. *Journal of Ardabil University of Medical Sciences*. 2017;17(4):487-96.
16. Mahboubi M, Kazempour N, Taghizadeh M. In vitro antimicrobial and antioxidant activity of *Vaccinium arctostaphylos* L. extracts. *Journal of Biologically Active Products from Natur*e. 2013;3(4):241-7.
17. Karimian R, Lahouti M, Davarpanah SJ. Effects of different concentrations of 2, 4-d and kinetin on callogenesis of *Taxus brevifolia* Nutt. *Journal of Applied Biotechnology Reports*. 2014;1(4):167-70.
18. Rashmi R, Trivedi MP. Effect of various growth hormone concentration and combination on callus induction, nature of callus and callogenic response of Nerium odorum. *Applied Biochemistry and Biotechnology*. 2014;172(5):2562-70.
19. Farjaminezhad R, Garoosi G. Improvement and prediction of secondary metabolites production under yeast extract elicitation of *Azadirachta indica* cell suspension culture using response surface methodology. *AMB Express*. 2021;11(1):43.
20. Chang C-H, Chiu H-F, Han Y-C, Chen IH, Shen Y-C, Venkatakrishnan K, et al. Photoprotective effects of cranberry juice and its various fractions against blue light-induced impairment in human retinal pigment epithelial cells. *Pharmaceutical Biology*. 2017;55(1):571-80.
21. Ziemlewska A, Zagórska-Dziok M, Nizioł-Łukaszewska Z. Assessment of cytotoxicity and antioxidant properties of berry leaves as by-products with potential application in cosmetic and pharmaceutical products. *Scientific Reports*. 2021;11(1):3240.
22. Michalska A, Wojdyło A, Honke J, Ciska E, Andlauer W. Drying-induced physico-chemical changes in cranberry products. *Food Chemistry*. 2018;240:448-55.
23. Vinson JA, Zubik L, Bose P, Samman N, Proch J. Dried fruits: excellent in vitro and in vivo antioxidants. *Journal of the American College of Nutrition*. 2005;24(1):44-50.
24. Miletj N, Popovi B, Mitrovj O, Kandic M, Leposavj A. Phenolic compounds and antioxidant capacity of dried and candied fruits commonly consumed in Serbia. *Czech Journal of Food Sciences*. 2018;32:360-8.
25. Jurikova T, Skrovankova S, Mlcek J, Balla S, Snopek L. Bioactive compounds, antioxidant activity, and biological effects of European cranberry (*Vaccinium oxycoccos*). *Molecules*. 2019;24(1):24.
26. Gudžinskaitė I, Stackevičienė E, Liaudanskas M, Zymonė K, Žvikas V, Viškelis J, et al. Variability in the qualitative and quantitative composition and content of phenolic compounds in the fruit of introduced American cranberry (*Vaccinium macrocarpon* Aiton). *Plants*. 2020;16;9(10):1379.
27. Jin D, Liu T, Dong W, Zhang Y, Wang S, Xie R, et al. Dietary feeding of freeze-dried whole cranberry inhibits intestinal tumor development in Apc(min/+) mice. *Oncotarget*. 2017;8(58):97787-800.
28. Kraujalytė V, Venskutonis PR, Pukalskas A, Česonienė L, Daubaras R. Antioxidant properties and polyphenolic compositions of fruits from different European cranberrybush (*Viburnum opulus* L.) genotypes. *Food Chemistry*. 2013;141(4):3695-702.
29. Yu H, Kim D-J, Choi H-Y, Kim SM, Rahaman MI, Kim Y-H, et al. Prospective pharmacological methodology for establishing and evaluating anti-cancer drug resistant cell lines. *BMC Cancer*. 2021;21(1):1049.
30. Lefranc F, Tabanca N, Kiss R. Assessing the anticancer effects associated with food products and/or nutraceuticals using in vitro and in vivo preclinical development-related pharmacological tests. *Seminars in Cancer Biology*. 2017;46:14-32.