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Investigating and Comparing the Effect of *Portulaca oleracea*, *Vaccinium myrtillus*, and *Berberis vulgaris* on Oral Squamous Cell Carcinoma in an Vitro Study

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Abstract: Background and Aim: Oral squamous cell carcinoma (OSCC) is a growing health problem worldwide, for which standard treatment strategies have failed to increase its recovery rate significantly. In recent decades, many studies have investigated herbal or natural compounds for the prevention and treatment of OSCC. The present study was performed to investigate the effects of the extract of *Portulaca oleracea* seed, *Berberis vulgaris*, and *Vaccinium myrtillus* fruit on the OSCC. Experimental procedure: Firstly, the herbal samples were purchased from the medicinal plant market and each one of the samples was extracted. Afterward, SCC-15 cells were purchased from the Iranian Biological Resource Center. MTT test was used to evaluate cell proliferation and to measure the transcription rate of ki67, miR-4492, and miR-877-5p genes using Real-time PCR. Finally, the western blot test was used to measure the translation rate of b-cell lymphoma 2 and matrix metalloproteinase-2 genes. Quantitative variables of the present study were described as mean and standard deviation and the three-way ANOVA was used via SPSS ver. 25. an independent t-test was used. P-value< 0.05 was considered a statistically significant level. Results and Conclusion: Briefly, the findings of the present study showed that all three extracts had significant effects on growth suppression, apoptosis induction, and OSCC metastasis. Also, the triple (PVB) combination and Portulaca-Berberis (PB) combination were shown to be the most effective. This result indicates the anti-cancer synergistic effect of these extracts especially P and B, on suppressing OSCC, which should be confirmed in future animal and human studies.

Keywords: Herbal Compound, Apoptosis, Cell Proliferation, Neoplasm Metastasis, Micro RNA

1. Introduction

1.1. Purpose & Definition

Oral squamous cell carcinoma (OSCC) is considered as the most common malignant cancer of the Oral cavity [1]. While some cancer treatment protocols such as surgery, radiation therapy, and chemotherapy are effective and useful for this disease, drug resistance and toxicity are the known problems in patients with OSCC yet [2]. Besides, metastasis is unavoidable during the course of treatment of this disease, which leads to poor prognosis and high mortality in patients with OSCC [3].

Nowadays, natural and herbal medicine is becoming increasingly popular worldwide, and evidence suggest that medicinal plants are the unlimited resources of these medicines. World Health Organization (WHO) estimated that herbal medicine-based care currently is the most common source of primary health care for 75-80% of the world's population [4].

In recent decades, many studies have investigated several natural and herbal elements for the prevention and treatment of OSCC such as Resveratrol [5], *Emblica officinalis [6]*, Oridonin [7], Black raspberry [8], and Curcumin [9].

The present study investigated and compared the effects of purslane seed extracts and compounds and two native Iranian fruits containing large amounts of anthocyanins: berberis vulgaris (barberry) and vaccinium myrtillus (bilberry) on cell proliferation, survival, and translation rate of MMP2 and BCL2 genes, to investigate the effect of cell invasion and apoptosis as well as the transcription rates of Ki67, miRNA4492, and miRNA877-5p genes, which are effective on the process of OSCC carcinogenesis and metastasis.

1.2. Literature Review

Purslane (*Portulaca oleracea L.*) is a well-known herb in the world that is not only used as an edible herb, but also as a traditional medicinal herb. Accordingly, this herb consists of flavonoids, pigments, polysaccharides, fatty acids, etc. Moreover, considering its high omega-3 content, purslane seed oil is expected to exhibit a high antioxidant activity [10, 11]. Due to the neuroprotective and anti-hypoxic effects of this herb, it is known as a longevity food and a natural antibiotic [11]. Also, Farshori et al. found anti-cancer properties of purslane seed extract on HepG2 human liver cancer cells. The results of this study showed that its seed extract can reduce the morphology and adhesion capacity of HepG2 cells [12].

Anthocyanins are a group of flavonoid compounds that, as secondary metabolites in plants, play a protective role against cancer and heart disease [13]. In this regard, Raspberry fruit and the fruits studied in this study (*Vaccinium myrtillus* and *Berberis vulgaris*) contain this metabolite.

Bilberry (*Vaccinium myrtillus L.*) is known as one of the richest sources of anthocyanins and polyphenols [14]. Among the ethanolic extracts of 10 edible berries, bilberry extract was the most effective one on the inhibition of the growth of HL60 human leukemia cells and HCT116 human colon cancer cells in vitro [15].

Barberry (*Berberis vulgaris*) is playing a prominent role among medicinal plants for more than 2,500 years. Correspondingly, previous studies showed that berberis has strong antioxidant and cytotoxic effects that can induce apoptosis [16]. Currently, anticancer activities of berberine (BBR), as the active component of berberis, has been reported in a number of cancers, including hepatoma, prostate cancer, glioblastoma, ovarian cancer, leukemia, and breast cancer [17]. In addition, another study showed that both berberine chloride and barberry ethanolic extracts can inhibit the growth of breast, liver, and colon cancer lines (MCF7, HepG2, and CACO-2, respectively) in a dose-dependent manner at different intervals [18].

It can be said that disrupted cell proliferation and cell death are the two main predisposing factors of cancer progression in almost all cases. Therefore, the growth and progression of a neoplasm can be assessed by examining the proliferation of cancer cells as well as the viability of these cells using the MTT test [19]. Notably, the MMP-2 gene produces a protein enzyme in the body. Moreover, one of the most important functions of this enzyme is breaking down the type IV collagen, which is known as a major structural component of the basement membrane. Also, some studies have shown that MMP-2 can induce invasion to lymph nodes and bone in OSCC [20, 21].

The Bcl-2 gene plays a role in inducing and inhibiting apoptosis. Accordingly, some of its members are involved in inhibiting apoptosis by inhibiting the synthesis of caspases. Also, the balance between inducers and inhibitors determines the apoptosis pathway and cell survival. If Bcl-2 protein gene expression reduces, the process of programmed cell death (apoptosis) occurs via activating the tumor suppressors such as P53 [22]. The ki67 expression is strongly associated with the proliferation and growth of tumor cells, so it is widely used in routine pathological research as a proliferation marker. The expression of this gene is significantly higher in malignant tumors with poor differentiation compared to normal tissue, which is recognized as a prognostic factor [23].

MicroRNAs are non-coding short molecules playing several known roles in cell growth, proliferation, and regulation of vital pathways for the progression of many human cancers [24, 25]. Since some miRNAs are directly involved in carcinogenesis, miRNAs expression patterns can be considered as strong indicators for the diagnosis, prognosis, and evaluation of the treatment response in a variety of cancers like OSCC. Besides, miRNAs may be considered as a new strategy for the prevention and treatment of OSCC [26, 27]. In the present study, two miRNAs were used, as miRNA877-5p and miRNA4492. According to the latest articles, the expressions of the two above-mentioned miRNAs is OSCC cells are significantly higher and lower than healthy tissue, respectively (p-value=0.0003, p-value=0.0004), which are considered as a part of the treatment response markers along with previously approved markers [28]. Since MIR4492 is downregulated in OSCC cells and is considered as a tumor suppressor gene, and miRNA877-5p is upregulated and considered as an oncogene, we expected an increased transcription rate for miRNA4492 and a decreased transcription rate for miRNA877-5p transcription, if the treatments were effective.

2. Methods

2.1. Herbs

At first, the bilberry (*Vaccinium myrtillus*), barberry (*Berberis vulgaris*), and purslane (*Portulaca oleracea* seed) samples were purchased from the medicinal plants market and were then identified in Herbarium of the School of Pharmacy. Afterward, Herbarium code was assigned to each one of them. In this regard, Herbarium codes of bilberry, barberry, and purslane were PMP-2670, PMP-2671, and PMP-2669, respectively. Subsequently, each one of the samples was extracted by 70% ethanol using maceration method. Later, the extracts were concentrated by a rotary device and dried in a vacuum oven. Total phenol and flavonoid levels of the extracts

were then measured using spectrophotometry method based on gallic acid and quercetin absorption/concentration curve.

2.2. Cells

After preparing the extracts, SCC-15 cells were purchased from the Iranian Biological Resource Center. SCC-cells were also kept in Dulbecco's Modified Eagle Medium (DMEM), and processed with 10% fetal bovine serum (FBS), 100 mg⁄ml penicillin-streptomycin, 4 mg⁄ml hydrocortisone, and 2.5 mg⁄ml fungizone. Afterward, the cells were incubated in a humid atmosphere containing 5% CO_2 and 95% air at 37°C.

2.3. Cell Proliferation

MTT assay was then used to evaluate the effects of *Portulaca oleracea* (P), *Berberis vulgaris* (B), and *Vaccinium myrtillus* (V) on the proliferation ability of tumor cells. Correspondingly, the HEK293T cell line (a derivative of human embryonic kidney 293 cells) was used as the normal cell line in the MTT assay, and the same SCC-15 cell line was also used in other tests as a gene-expression inhibitor with no treatment. Finally, statistical comparisons were performed on the average effect of the extracts and therapeutic compounds on the variables. In order to determine the half maximal inhibitory concentration (IC50), as a drug concentration, in which 50% of the cells were viable, MTT assay was performed. The results were presented as average and expressed as a percentage of the control group. The IC50 values obtained from P, B, and V extracts were equal to 0.1, 10, and 2mg / ml, respectively.

2.4. Real-time PCR

Real-time PCR was used to measure the expression levels of ki67 genes as well as the studied miRNAs. Tumor cells were cultured with 80% confluence in 6-well plate. Finally, the resulting cells were extracted as well as the total RNA from the tumor cells. Also, the RNA concentration and purity were measured by measuring the absorption rate at 280 nm and 260 nm, respectively. The expression levels of ki67 and miRNA genes were determined using one-step RT-PCR kit. The total RNA (1μg) was translated into c DNA in terms of the manufacturer's protocol using reverse transcription. Also, the specific primer sequence was then determined.

2.5. Western Blot

Western blot was performed to analyze MMP-2 and BCL-2 expression. Defined proteins were acquired from cells by protein extraction kit (Biorad laboratories 163-2086). Proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were fixed and Then proteins transferred onto PVDF membrane. The membranes were blocked in TBST (Tris-buffered saline containing 0.05% Tween-20 buffer) containing 5% non-fat milk, and then incubated with Anti-Bcl-2antibody (ab59348), Anti-beta Actin antibody (ab8227), Human MMp-2 antibody (WBC025) in ratio 1/100 overnight at 4°C followed by 1 h incubation with (HRP) Horseradish peroxidase secondary antibody.

2.6. Statistical Analysis

Quantitative variables of the present study were described as mean and standard deviation with the assumption of normal distribution. To investigate the effects of P, V, and B elements on the expressions of miRNA4492, miRNA877-5p, and ki67genes, the three-way ANOVA was used via SPSS ver. 25. Considering the significance of the interaction among the studied elements, independent t-test was used to investigate the effect of each factor in the subgroup of other factors. P-value< 0.05 was considered as the statistically significant level.

3. Results

In this section, the first letter of the name of the relevant extracts is presented in all tables and diagrams, so P is equivalent to *Portulaca*, V is equivalent to *Vaccinium*, B is equivalent to *Berberis*, PB is equivalent to *Portulaca* - *Berberis*, PV is equivalent to *Portulaca* -*Vaccinium*, VB is equivalent to *Vaccinium*- *Berberis*, and PVB is equivalent to *Portulaca* - *Vaccinium*-*Berberis*. Also, in all tests, except the MTT assay, the control group included the untreated OSCC cells and its number was considered to be 1 in all tests. Therefore, these extracts were effective considering the change in the response rate to higher or lower than 1. Moreover, to investigate the significance of these numbers, p-Value will be related to the comparison of the effects of extracts and compounds on each other based on statistical analysis.

3.1. Phenol & Flavonoid

The concentration of these metabolites was the highest in Vaccinium myrtillus and then Berberis vulgaris and Portulaca oleracea.

3.2. Cell Growth and Survival

In the MTT assay, we had two cell-line groups, as OSCC and HEK293T. Cell growth was similar for both untreated groups; however, after exposure to the extracts, the growth and survival rates in the OSCC group were less than HEK293T.

In the PVB and PB groups, greater anti-growth effect was exhibited compared to other compounds [Figure 1]. However, there was no significant difference among these three.

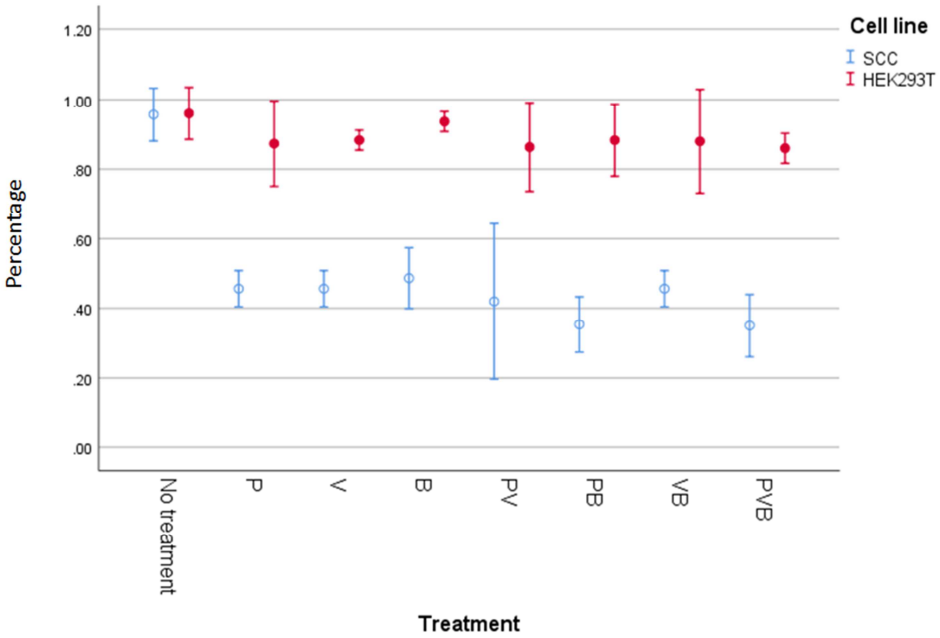

Figure 1. *Cell growth and survival rate (mean, 95% confidence interval) in the SCC cell-line compared to hek293t after exposure to natural compounds. P: Portulaca oleracea, V: Vaccinium myrtillus, B: Berberis vulgaris.*

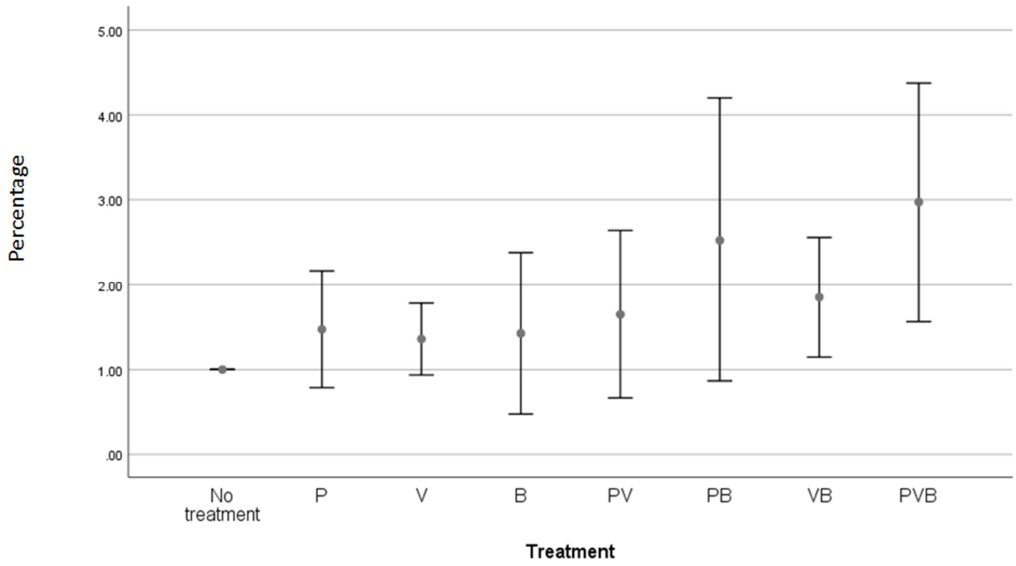

Figure 2. *Transcription rate (mean, 95% confidence interval) of the miRNA4492 gene in SCC cells after exposure to natural compounds. P: Portulaca oleracea, V: Vaccinium myrtillus, B: Berberis vulgaris.*

3.3. Bcl-2

All the extracts have reduced the Bcl2 translation, with PVB, PB, and P extracts showing more effectiveness than other compounds, respectively [Table 1].

3.4. MMP-2

P, PB, and PVB were the most effective extracts and VB was the least effective extract on the inhibition of the translation of MMP-2 [Table 1].

The quantitative results of the western blot test (using Gel analyzer software) for MMP-2 and BcL-2 variables are as follows in Table 1.

Table 1. *Translation rates of MMP2 and BCL2 genes in SCC cells after exposure to natural compounds (in percentage compare to control group).*

Treatment	BCL2 protein	MMP2 protein
No treatment	1.00	1.00
P	0.50	0.40
V	0.60	0.50
B	0.90	0.60
PV	0.60	0.50
PB	0.40	0.40
VB	0.80	0.90
PVB	0.30	0.40

P: Portulaca oleracea, V: Vaccinium myrtillus, B: Berberis vulgaris

3.5. mi-RNA 4492

The mi-RNA 4492 expression rate was higher in SCC cells treated with all the extracts compared to the control group, and among these extracts, PVB was found as the most effective combination, followed by PB, VB, and PV, respectively [Figure 2]. Also, B extract in PVB combination has been more effective on the miRNA-4492 expression as compared to PV (p-value=0.03).

3.6. mi-RNA877-5p

The gene expression level was less than one (control), with in PVB and PB groups, which indicate the greatest reduction and thus exhibit the greatest effectiveness, respectively. However, there was no statistically significant difference between PVB and PB combinations in terms of their effects on this variable [Figure 3]. In other word, the addition of V extract had no additional effect on the mi-RNA877-5p expression. Also, B extract in PB and PVB combinations had a significant effect on the expression of miRNA877-5p gene compared to P extract (p-value=0.02) and PV (p-value=0.03).

3.7. ki67

regarding this gene, which represents the ability of remote proliferation and metastasis, PVB again outperformed all the other compounds, and significantly reduced the ki67 expression compared to VB (p-value: 0.01 <0.05), followed by PB, PV, and VB extracts, respectively [Figure 4]. In addition, the PVB compound was more effective on transcribing Ki67 compared to the PB compound. In other word, the V extract had a significant effect on the transcription of ki67 (p-value=0.02).

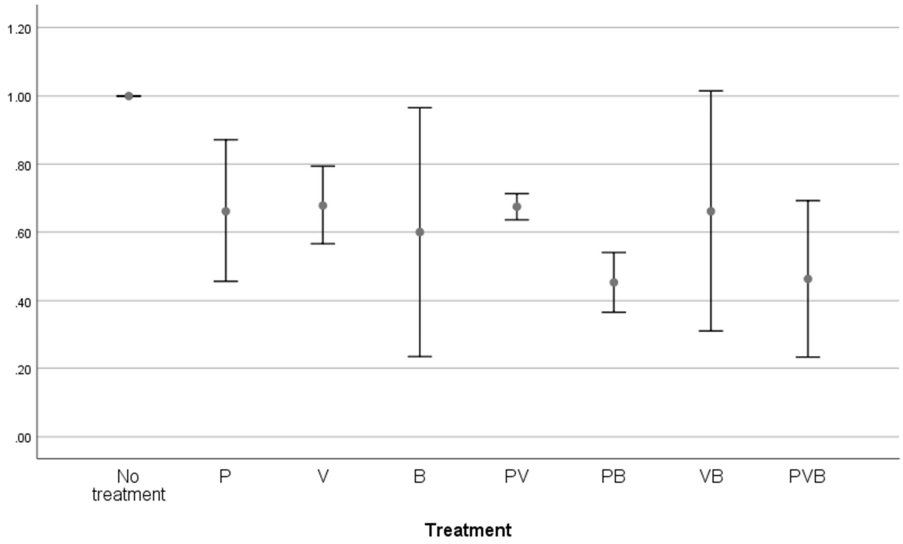

Figure3. *Transcription rate (Mean, 95% confidence interval) of the miRNA877-5p in SCC cells after exposure to natural compounds. P: Portulaca oleracea, V: Vaccinium myrtillus, B: Berberis vulgaris.*

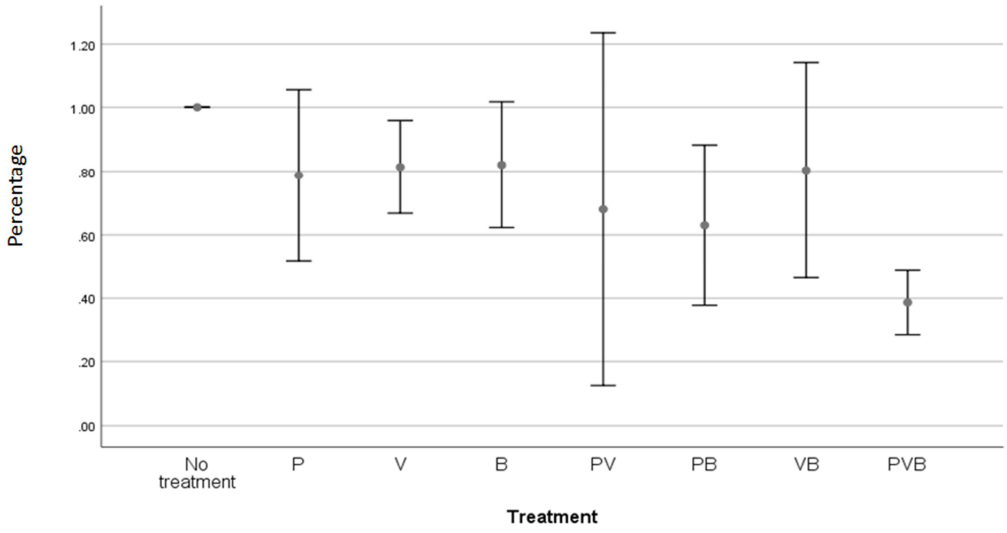

Figure 4. *Transcription rate (mean, 95% confidence interval) of the ki67 gene in SCC cells after exposure to natural compounds. P: Portulaca oleracea, V: Vaccinium myrtillus, B: Berberis vulgaris.*

4. Discussion

In summary, the present in vitro study investigated and compared the effect of Purslane, barberry fruit, and bilberry extracts, which are native Iranian fruits. The results show that all these elements had significant effects on suppressing growth, invasion, and induction of cell apoptosis in OSCC.

To the best of the author's knowledge, there has been no study on synergistic effect of these three herbal extracts on OSCC, so far. Therefore, these results cannot be compared with completely similar studies.

The effect of Purslane extract on suppressing OSCC growth and proliferation is consistent with GaiGuo and Farshori's studies, which confirmed the same effect on cells in cervical, esophagus, breast, and liver cancers [11, 12]. Barberry extract-induced cell growth and survival in SCCO are also consistent with the studies performed by E Abd EL-Wahab and Ren who found the same effect on cells in breast, liver, colon, and esophageal cancer cells [18, 29]. Finally, the results of a study by Tumbas that confirmed the effect of bilberry extract in preventing the growth of breast, cervical, and colon cancers [30], are consistent with the results of the present study, suggesting the effect of this extract on reducing the growth and cell survival of SCC cells. Moreover, the present study investigated the synergistic effect of these elements on cell growth and survival, and the results reveal that all these three extracts and their compounds were significantly effective in this regard. However, MTT assay showed no significant difference among these three extracts in terms of their effects. Also, a significant decrease in ki67-gene expression showed a positive effect of all these elements, especially PVB combination (more than PB) on reducing the proliferation and metastasis rate. In other word, it can be said that V and P extracts were more effective on reducing the Ki67 expression compared to the other one.

The effect of purslane extract on cell death induction in SCC is consistent with Hassan A.'s study on liver cancer cells [31]. Accordingly, in a mouse model of breast cancer, Hussein A. showed that the purslane extract reduced the tumor size [32]. Moreover, Hoshyar and Ren showed in their study that barberry extract induces apoptosis in breast and esophageal cancers [29, 33]. Also, Katsube *et al.* indicated that bilberry extract was effective on inhibiting leukemic cells and subsequently the inhibition of the growth of colorectal cancer cells through inducing apoptosis [15]. Nguyen *et al.* also reported the effective dose-dependent function of bilberry extract on breast cancer cells by inducing apoptosis [34], which is consistent with the present study, indicating that PVB, PB, and P extracts, play significant roles in inhibiting translation of BCL2, which is an indicator of cell apoptosis inhibition, as compared to other elements, respectively. In other words, the present study showed that PVB, P, and then B were more effective on inducing apoptosis.

The present study also investigated the effect of these compounds on the translation rate of MMP2 protein, as an indicator of cell invasion. The results show that P, B, and PVB extracts were more effective on inhibiting the translation of this protein, and VB had the least effect in this regard. Moreover, it investigated the transcription rates of two miRNAs in OSCC that indicate tumor suppression or carcinogenicity; however, up to now, there have been no similar studies examining the effect of these compounds on cancers through miRNAs. The results of the present study are consistent with the previous results of the study in both cases. In addition, PVB and PB were found to be more effective on the transcription rate of the miRNA4492 gene as compared to other extracts and compounds in increasing expression from a tumor suppressor gene. However, there was no significant difference between PVB and PB in this regard, which means that the addition of V extract had no significant effect on the expression of this gene. Nevertheless, as compared to others, B extract had a better positive synergistic effect on this variable. The PVB group was also significantly more effective on increasing the miRNA4492 gene expression compared to the VB group (p-Value=0.04). This result could indicate the strong effect of P extract on miRNA4492 as well as the good synergistic effect of PB.

The reduction in miRNA877-5p gene expression showed that the PVB and PB were significantly more effective than the other compounds or the use of a single element alone. In other word, PB or PVB were more effective on preventing the expression of this proto oncogene. The PB was also significantly more effective than P on reducing the transcription of the miRNA877-5-5 gene, indicating a good effect of B extract on this gene as well as the synergistic effect of PB. However, PB and PVB had no significant effects on this miRNA, which may indicate that V extract was less effective on the studied miRNAs. Moreover, gene transcription and cell growth were not significantly different between B and VB groups as well as P and PV groups, which may indicate a lower effect of V extract on this variable or poor synergy of V extract along with B or P.

Therefore, as shown by the results of the tests, despite the positive effect of *Vaccinium myrtillus* (V) on reducing carcinogenesis and increasing tumor suppressor factor in all these variables, this effect was less than the other extracts and this extract could only exhibit a better synergistic effect in PVB combination on inhibiting ki67 as compared to PB compound as well in PV combination as compared to BV. This result is in contrast to (inconsistent with) total flavonoids and phenols measured in these extracts before exposure to cells (Vaccinium > Berberis > Portulaca oleracea) and our study showed that other compounds in Portulaca oleracea and Berberis compared to flavonoids and phenols are probably more effective on oral squamous cell carcinoma.

Notably, various factors can affect the phenolic profile of plants including genotype, geographical location, altitude, climatic conditions, technological measures, and time. Previous studies showed that bilberry that grow in places with powerful sunshine contains higher amounts of total sugars, anthocyanins, flavanols, and hydroxycinnamic acids compared to the one growing in places with low sunshine radiant [35]. Nguyen *et al.* (2009) showed that bilberry extract

affects breast cancer cells in a dose-dependent manner and is able to prevent the growth and proliferation of breast cancer cells by inducing apoptosis (IC50=0.3-0.4 mg / ml). However, to affect the organization of microtubules and the cell cycle directly, higher bilberry concentrations (0.5-1 mg / ml) are needed [34]. Accordingly, in the present study the minimal inhibitory concentration (MIC) was used, as 2 mg.

To ensure the effects of Vaccinium extract, we should determine and take into account more details about the genotype, geography, time, and technological conditions (such as growth in a bright environment) of this fruit. Although the anti-cancer effects of this fruit on OSCC have been confirmed in the present study, the lower effect of this fruit extract as compared to the other extracts may be justified due to its growth and technological sensitivities.

1) In this study, by considering that the herbs were prepared from the market of medicinal plants and then identified and extracted by the botanist, we were not able to determine the exact geographical location of their origin in nature, which can be one of the limitations of the present study. Therefore, it is suggested that, in future studies on the use of vaccinium myrtillus extract and to ensure its stronger effects, more details about genotype, geography, time, and other factors (such as growth in a bright environment) of this fruit need to be specified and observed.
2) The results obtained in in vivo environment of the present study should be confirmed and if these results are confirmed, the effective and safe forms of drug compounds should be taken into account.
3) Further studies are recommended to investigate the effects of these herbs on the cell cycle, to allow performing relevant interventions and thus preventing the development of precancerous lesions to cancerous lesions.
4) Further studies are needed to discover other mechanisms (such as microRNAs, mediators, and cytokines) used by these compounds to affect OSCC.
5) It is suggested to conduct other studies to compare effectiveness of secondary metabolites of these extracts on oral squamous cell carcinoma.

5. Conclusion

The present study showed that *Portulaca oleracea, Vaccinium myrtillus, and Berberis vulgaris* extracts can inhibit the growth of cancer cells and significantly reduce the transcription rates of Ki67 and miRNA877-5p genes, which represent cell growth, invasion, and carcinogenicity. Also, they can increase the transcription rate of the miRNA44492 tumor suppressor gene. Moreover, they reduce the expressions of BCL2 and MMP2 proteins, which consequently inhibit local invasion and reduce apoptotic inhibitors. The present study also revealed that PVB combination was the most effective one, followed by PB in all variables (equal or more effective than P or b alone), which shows the good synergistic effects of purslane and barberry extracts on controlling oral cancer. At the same time, vaccinium myrtillus extract in PVB or PV combination was significantly effective on the inhibition of the ki67 gene expression. Therefore, it can be used as a complementary preventive and therapeutic method in the suppression of OSCC after confirming the above-mentioned effects in further studies and determining the prescription details.

Conflict of Interests

All the authors do not have any possible conflicts of interest.

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