Effect of Nasturtium Extract on Oral Cancer

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Abstract

Background: Considering the global prevalence of cancers and the complications of common cancer treatments, there is growing interest in using medicinal herbs to complement cancer treatments and reduce treatment's side effects. Therefore, we investigate the effect of the extract of Nasturtium on the viability of oral cancer cells.

Materials and Methods: In this experimental study, we prepared aqueous extract from Nasturtium leaves and human oral cancer cells (OCC-24) and normal fibroblast cells (HF2FF cell line) from a cell bank. Then the toxic effect of different concentrations of the extract on cell viability after 24–48 hours of exposure was investigated with the methylthiazol tetrazolium assay. Ultimately, the optical density was measured at 570 nm by an Elisa Reader. Analysis of inhibitory concentration 50 (IC50) was also performed. The data were analyzed by paired Student's t-test and one-way analysis of variance.

Results: Data showed that the extract had statistically significant anticancer effects in concentrations above 0.125 mg/ml for 24-hour exposure and in concentrations above 0.5 mg/ml for 48-hour exposure (p-value <0.05). Also, this extract had an adverse effect on the viability of normal cells; however, this effect occurred in high doses of the extract (p-value <0.05). Analysis of IC50 criteria indicates that with increasing time, a higher concentration of the extract is required to inhibit the viability of cancer cells.

Conclusion: Because of the results, this aqueous extract can be suggested as a potential therapeutic agent in oral cancer. The best concentration of the extract was found to be 1 mg/ml.

Keywords: Extract, MTT, Nasturtium, oral cancer

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common oral cancer with varied clinical manifestations. Despite the accessibility of therapeutic methods such as surgery, radiotherapy, and chemotherapy in most countries, the 5-year survival rate of oral cancer, especially OSCC, remains below 50%.[1,2] Therefore, new treatments such as the usage of natural products and phytochemicals derived from plants are needed to prevent the development of this cancer with less systemic toxicity and side effects in humans.[3]

Cruciferous vegetables are part of the Brassica genus of plants which includes watercress, broccoli, cabbage, and so on, containing substances that have anti-cancer effects.[4]

Watercress (WC) with the scientific botanical name Nasturtium officinale is a plant being explored for multiple health benefits because it is full of ingredients such as glucosinolates, carotenoids, polyphenols, and vitamin C, vitamin A, and α-tocopherol.[5]

Glucosinolates are valuable secondary metabolites, mainly found in the Brassicaceae family. Isothiocyanates (ITCs) were characterized as small organic compounds synthesized as glucosinolates with R–N = C = S functional groups. They

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were converted into an active form when the plants are digested. ITCs have an anti-cancer effect. This effect is caused by inhibiting cell growth and proliferation through inhibiting mitosis or by inducing apoptosis in human tumor cells.

More than 20 ITCs have been identified with anti-carcinogenic properties. Among these, benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), and sulforaphane (SFN) are the important ones.

BITC, PEITC, and SFN suppress the tumor growth of various cancer cell lines in the breast, brain, lung, oral, and so forth.[6-8] It should be noted that in watercress, PEITC is more abundant than other ITCs.[6]

In confirmation that ITCs have anti-cancer effects, in 2014, a study that investigated the molecular mechanism and anti-cancer potential of PEITC in oral squamous cell carcinoma (OSCC) cells with various p53 statuses showed the following results: PEITC was able to (1) inhibit cell growth in cell lines such as OC2, SCC4, and SCC25 cells in a dose-dependent manner with low effects on normal cells, (2) arrest G2/M phase, and (3) induce apoptosis by reducing the expression of Bcl-2 and Mcl-1, released mitochondrial cytochrome c, and activated caspase 3 and PARP cleavage.[9]

Another study in 2012 investigated the anti-cancer effect of PEITC on OSCC (HSC-3 cell line) and its mechanism of action. In this study, a flow cytometric assay was used for viability assessment. Also, evaluation of changes in the cell cycle and the level of proteins involved in apoptosis determined by Western blotting in cells after exposure to PEITC was performed. Their results indicated that PEITC effectively inhibited the cancer cells' growth by induction of G0/G1 phase arrest and causes cell apoptosis by affecting the proteins and markers involved in this cellular cascade. This study introduced PEITC as an anti-cancer agent in the treatment of oral cancer.[10]

In a research in 2022, a study was conducted on the effects and properties of watercress on melanoma. This study mentioned that watercress is a rich source of PEITC, with an anti-oxidant capacity. The results of this study showed that the cytotoxicity potential of watercress was considerably higher in human malignant melanoma (A375 cell line) than in non-melanoma epidermoid carcinoma (A431) and keratinocyte cells (HaCaT). Also, in the investigation of oxidative stress and induction of apoptosis in the edible and non-edible parts of watercress, it was found that the edible parts such as the leaves of the plant have stronger effects than the non-edible parts such as stems.[11]

In 2019, a study investigated the role of ITC on melanoma and its treatment. In the text of this article, it is stated that ITC present in cruciferous plants can lead to cell cycle growth arrest and apoptosis induction in human melanoma.[12]

In a review article published in 2021, it was stated that the crude extract of the watercress shows a high level of anti-oxidant ability and has the ability to inhibit the three stages of initiation, proliferation, and metastasis in the carcinogenesis process in the laboratory.[13]

Considering the medicinal properties of watercress and because of the existence of very limited articles on the anti-carcinogenic effect of watercress on oral cancer, the present study was designed to investigate the possible anti-cancer effects of the aqueous extract of this plant.

MATERIALS AND METHODS

Watercress leaves were acquired from the Iranian Institute of Medicinal Plants (Jahad Daneshgahi) in Tehran. The leaves were washed with distilled water and then air-dried at room temperature in the dark.

To prepare the aqueous extract, 20 g of leaves was milled and then mixed with 500 ml of boiling water, and the resulting mixture was passed through a filter three times to separate the solid fraction.[14] The aqueous extract was prepared at eight different concentrations (0.06, 0.125, 0.25, 0.5, 1, 2, 4, and 8 mg/ml) in order to improve the generalizability and accuracy of findings.

The human oral cancer cells with cell number (IBRC C11068) and code (OCC-24) were acquired from the cell bank of the Iranian Biological Resource Center (this cell line is from the sapiens species and belongs to a 41 years old male; it is not genetically engineered and has an epithelial-like morphology). Normal fibroblast cells (HF2FF cell line) were used as the control group. The cells were obtained using the enzymatic isolation method (collagenase type 1), and then CD326 (EpCAM) positive cells were isolated using the magnetic-activated cell sorting (MACS) technology to check the morphology and cell health and ensure the absence of bacterial, mycoplasma, or fungal contamination. All procedures were performed in a laboratory fume hood in a culture room under sterile conditions, and cells with a biological capacity of more than 90% were used to experiment.[15]

The toxicity effect of the aqueous extract on the viability of cancer cells and control cells was measured by applying different concentrations of the extract to the rows of plate wells for 24 and 48 hours and then conducting the MTT (methylthiazol tetrazolium) assay (It should be noted that in this study, the idea of determining different times was taken from previous studies on the anti-cancer effects of this plant extract on different cancers).[15-18]

The lack of exposure of cancer and control cells to the extract (i.e., exposure to zero concentration) was considered the negative control.[15,16] For the MTT assay, 50 μL of the MTT reagent was added to the plates. About 5000 cancer cells were cultured in four rows of a 96-well flat-bottom plate with 50 μL of the medium and kept in an incubator for 24 hours under the same conditions. The top, bottom, leftmost, and rightmost wells were kept empty (blank). The reduction of tetrazolium salt and the production of formazan crystals were easily detectable under a microscope. In this

assay, the amount of pigment produced is directly proportional to the number of metabolically active (lives) cells. Because formazan crystals are water-insoluble, they must be solved in a solvent like dimethyl sulfoxide before colorimetry. The method of analyzing the results of viability tests such as the MTT assay is that after reading the optical density of the solution at 570 nm with an ELISA reader, it is obtained from the absorption of repeated houses for each dose. It should be noted that the amount of absorption of each well is reduced from the amount of absorption of the blank well. Finally, the percentage of cytotoxicity and cell viability was calculated according to the mean absorbance of toxicant-treated cells into a mean absorbance of the negative control.

% viability = (mean experimental absorbance/mean negative control absorbance) × 100.[19]

After the calculation of cell viability percentage, the concentration of aqueous extract giving 50% inhibition on the OCC-24 cell line (IC50 or half-maximal inhibitory concentration is the concentration that provides 50% reduced cell growth compared to the control) was determined at various incubation times.

Also, the value of the IC50 index, which determines the dose of the drug to inhibit the survival of 50% of the cells exposed to different doses, by drawing a logarithmic regression line for the dependent variable of the number of healthy cells remaining after exposure to the independent variable of different doses in time 24 hours and 48 hours is calculated through the following relations:

Y = a + b*logx

IC50 = (50-a)/b

The results of cell viability were determined as a mean using Microsoft Excel. Graph Pad Prism version 4.00 was used to calculate IC50. Statistical analysis was performed by paired Student's t-test, one-way analysis of variance (ANOVA), and repeated measures. *P* values less than 0.05 were considered statistically significant.[19]

RESULTS

After statistical analysis, in response to the objectives of this study, it can be said that the aqueous extract of the plant had anti-cancer properties. This property increased when increasing the concentration (p < 0.05) [Table 1]. To be more precise, the aqueous extract of watercress reduced the viability of cancer cells, an effect that was statistically significant at all dosages above 0.125 mg/ml for 24-hour exposure (p-value < 0.05) and at all dosages above 0.5 mg/ml for 48-hour exposure (p-value < 0.05).

According to IC50 investigation, it was found that this anti-cancer property has decreased when increasing time, which means that with increasing time, a higher concentration of extract is required to destroy at least 50% of cancer cells (IC50 of the extract for cancer cells was determined to be 3.12 mg/ml and 3.83 mg/ml in 24 and 48 hours, respectively [Table 1].

The results showed no significant relationship between the time alone and the mean percent number of surviving cells/viability% (p = 0.07), which is expectable. However, the mean percent number of surviving cells/viability % was found to be significantly related to the combination of time and extract dosage (p < 0.05).

However, the important point is that the aqueous extract of the plant had an adverse effect on the viability of normal cells, although this effect was observed at higher concentrations of the extract, which was statistically significant at dosages of 4 and 8 g/ml (p-value < 0.05) for 24 hours and at all dosages above 0.5 mg/ml for 48 hours (p-value < 0.05). In other words, this adverse effect increased when increasing the concentration. Also, based on IC50 investigation, it was found that this adverse effect has increased with increasing time, which means that with increasing time, a lower concentration of extract is

Table 1: Analysis of the effect of the aqueous extract of watercress on cancer and normal cells after 24 and 48 h (at *P*=0.05 level) and the corresponding IC50 values

Cell	Cancer cell						Normal cell					
Time	24 h			48 h			24 h			48 h		
Dosage mg/ml	*t*-test P	Viability %	IC50 mg/ml	*t*-test P	Viability %	IC50 mg/ml	*t*-test P	Viability %	IC50 mg/ml	*t*-test P	Viability %	IC50 mg/ml
8	0.000	17.0	3.12	0.000	20.6	3.83	0.003	76.5	14.03	0.001	62.2	10.29
4	0.000	27.1		0.000	28.7		0.007	83.7		0.001	77.7	
2	0.000	42.6		0.000	53.6		0.172	96.1		0.000	77.9	
1	0.000	54.9		0.000	70.2		0.226	97.2		0.004	88.0	
0.5	0.000	70.4		0.000	85.3		0.485	100.1		0.001	88.2	
0.25	0.000	78.5		0.091	98.3		0.238	102.6		0.052	96.5	
0.125	0.050	96.8		0.115	98.9		0.051	108.1		0.232	97.6	
0.06	0.335	99.3		0.391	99.7		0.051	108.4		0.483	100.1	
Positive control	0.00	5.4	-	0.00	3.7	-	0.00	5	-	0.00	4.3	-
Negative control	0.50	100.0	-	0.50	100.0	-	0.50	100.0	-	0.50	100.0	-

Positive control=complete medium. Negative control=Only cells without using any concentration of extract

required to destroy at least 50% of normal cells (IC50 of the extract for normal cells was determined to be 14.03 mg/ml and 10.29 mg/ml in 24 and 48 hours, respectively [Table 1].

Among the dosages that were significantly effective in killing cancer cells in 24 hours, it seems that the concentration of 1 mg/ml is the most ideal concentration used because in this concentration, 54.9% of cancer cells and 97.2% of normal cells survive and this concentration destroys approximately 50% of cancer cells and causes minimal damage to normal cells.

In general, the interpretation of the graph shows that by increasing the concentration of the extract, its effect on destroying cancer cells increases, and it is worth considering that this increase in the extract concentration will also have a destructive effect on healthy cells. This is even though these mentioned doses have a very little destructive effect on healthy oral cells [Diagram 1].

DISCUSSION

In this study, we investigated the anti-cancer effect of Nasturtium officinale on oral cancer cells using the MTT assay. The results of this study showed that the aqueous extract of this plant reduced the viability of the oral cancer cell line and this effect had an inverse relationship with exposure time and a direct relationship with the concentration of the extract. Our examination of IC50 and the combined effect of exposure time and extract concentration showed that as the exposure time increased, a higher concentration of the extract was needed to inhibit the viability of cancer cells.

In a study by *Yeh YT*,[9] investigations showed that PEITC has a dose-dependent cytotoxicity effect on oral cancer cell lines. This finding was consistent with the results of the present study regarding the anti-cancer effect of watercress extract on cancer cell lines. This result is a confirmation of the presence of the effective substance PEITC in this plant and its cytotoxicity effects on tumoral cells. Also, the growth inhibitory effect of the extract in the present research was directly related to the concentration of the extract, which was in line with the

Diagram 1: Comparative diagram of the distribution of the remaining cells exposed to different concentrations in 24 and 48 hours

mentioned study. It should be noted that negative effects on healthy cells have also been reported in both studies, and by controlling the concentration and limiting the concentration to 1 mg, minimal cytotoxicity can be left on normal cells.

Another investigation by *Chen PY*[10] already pointed out that PEITC induced cytotoxic effects on human oral squamous cell carcinoma (HSC-3 cells). These suggestions were based on some observations such as decreasing the viability of tumor cells in a dose- and time-dependent manner, inducing apoptosis by increasing pro-apoptotic proteins, and decreasing anti-apoptotic proteins. The decrease in the viability of tumoral cells because of the use of PEITC is a finding that was also mentioned in the present study that the aqueous extract of watercress reduced the viability of the oral cancer cell line and this effect was increased when the concentration of the extract was increased. Finally, according to the findings obtained in this study, Chen and his colleague suggested that PEITC can be an anti-cancer substance in the treatment of oral cancer, which can be somewhat extended to the use of watercress extract that contains PEITC. In this study, different concentrations of PEITC (0.5, 1, 2, 2.5, and 5 μM) were used for 24, 48, and 72 hours to check viability. The significant and important point in this study is the use of the effective substance PEITC, which can remove many cumulative effects caused by the presence of other elements in the plant and its extract, and to only investigate the anti-cancer effect of PEITC.[10]

In a study performed by *Kyriakou, S*,[11] the anti-cancer effects of extracts obtained from edible and non-edible parts of watercress on human malignant melanoma (A375), non-melanoma epidermoid carcinoma (A431), and non-tumorigenic keratinocytes (HaCaT) were evaluated. Findings indicated that the cytotoxicity effect of the extract obtained from the edible components was much stronger on melanoma, whereas non-melanoma epidermoid carcinoma and keratinocytes were more resistant to anti-tumor effects or less affected from the extract, respectively. In the present study, the edible parts of the plant were used to prepare the extract, whose anti-cancer results were much higher on cancer cells than on normal cells, a finding that was in line with the aforementioned study. In *Kyriakou, S*'s study, the anti-cancer effect of the extract decreased the viability of cancer cells in a concentration- and time-dependent manner. Also, the amount of PEITC obtained in the edible parts of the plant (1695 ± 100.46 μg/g dry watercress) was more than that in the non-edible parts (1002 ± 94.21 μg/g dry watercress), which confirms the results.[11]

In another research by *Mitsiogianni M*,[12] they examined the anti-cancer effect of PEITC on patients with oral cancer with a history of consumption of tobacco. Chewing tobacco and smoking are major risk factors for oral cancer. When tobacco is used, the procarcinogenic NNK (a nitrosamine compound present in tobacco) metabolically reacts with CytochromeP450 and then converts to a carcinogenic type. Carcinogenic NNK binds with DNA to form tumors. They found that PEITC acts
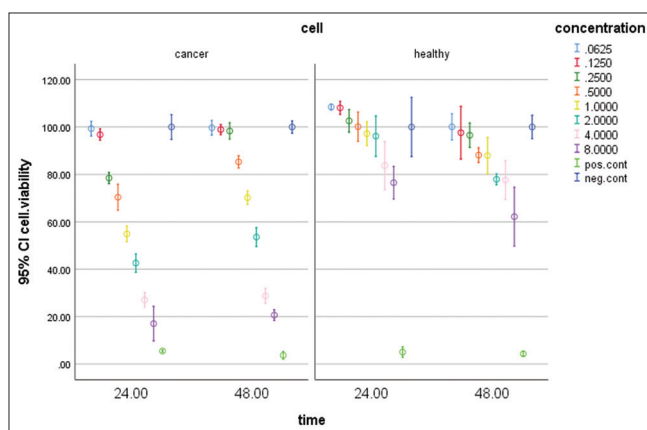
as an inhibitor of the metabolism of NNK with Cytochrome P450 because of a higher binding affinity between PEITC and Cytochrome P450 than NNK with Cytochrome P450. Therefore, the production of carcinogenic NNK that leads to cancer is prevented. This study, in agreement with the present study, emphasized the anti-cancer effect of watercress because of its PEITC compounds.[12]

Our findings also are consistent with the findings of a study conducted by Aggarwal *et al.*[20] about the interaction and relationship between p53 and PEITC in prostate cancer, where the anti-cancer effect of watercress was confirmed and attributed to its phenyl isothiocyanate content. These researchers stated that certain concentrations of this substance may have an anti-cancer effect on prostate cancer cells with different P53 mutations under certain exposure times. They attributed this anti-cancer effect to the re-activation of mutant p53s in this cancer. It should be noted that the effective dosage to achieve this effect in Aggarwal's study was 8 µM in 4 hours, which is not consistent with our findings but is nonetheless reasonable considering the type of cancer they studied (prostate cancer). Another study by *Rose P*[21] has also reported that PEITC disrupts the translation process of cancer cells by affecting important cell factors and inhibiting protein synthesis factors; for this reason, increased PEITC concentration is associated with decreased protein production and increased anti-cancer activity.[21] Other researchers have suggested that this substance plays an anti-cancer role by inhibiting hypoxia-inducible factor (HIF) transcription activity, which is a key regulator of malignant cell angiogenesis.[22,23] All of the above findings are consistent with our results regarding the anti-cancer effect of watercress extract.

A study founded by Sefidkan *et al.*[18] also investigated the anti-cancer effect of watercress extract nanocapsules with concentrations of 0.5–1.5 mg/ml on breast and colon cancer cell lines using the MTT assay. As in the present study, the findings of Sefidkan's study confirmed the anti-cancer effects of watercress. In that study, the anti-cancer effects were observed on both cell lines over 24 to 72-hour periods. After 3 days, the nanocapsules made with a concentration of 150 nM killed 72% of cancer cells and the total extract with the same concentration destroyed 60% of cancer cells. The study also reported that the effects were stronger on breast cancer cells than on colorectal cancer cells. The only point of inconsistency between the findings of our study and Sefidkan's study is that although we observed an inverse relationship between the anti-cancer effect and the exposure time, in that study, the anti-cancer effect increased with the exposure time and the highest kill ratio was observed after 3 days.[18] This discrepancy can be related to the difference between the two studies in terms of the type of cancer studied and the involved carcinogenesis mechanisms and mutations. Furthermore, the two studies differ in terms of the method of preparing the extract and the nanocapsules, which could be very important because the strength of the anti-cancer effect of the extract and its effective concentration very much depend on what solvent is used in the extraction process, which substances are extracted, and how much of each substance the extract contains.

In a study by Lara *et al.*,[24] these researchers examined the effect of watercress consumption on the growth of the Ehrlich tumor in animal models. A comparison of groups of subjects that were given the same amount of aqueous watercress solution at certain times showed that the daily consumption of the solution was able to suppress tumor growth, an effect that was attributed to the anti-tumor properties of the compounds contained in the plant. These results are also consistent with our findings regarding the anti-cancer effect of watercress extract. It should be noted that in this animal study, only one dose of the extract (0.05mg/ml) was used.[24]

In a study of Fallah *et al.*,[16] the anti-cancer effect of watercress extract on a breast cancer cell line using the MTT method was investigated. After examining the anti-cancer effect of different concentrations of the hydroalcoholic extract of the plant (0–2 mg/ml) in 24 to 72-hour periods, these researchers reported that the extract had a significant anti-cancer effect on the cell line, which was directly related to its concentration. They also found that the extract had a limited statistically insignificant (P-value >0.05) adverse effect on the control group (i.e., normal fibroblast cells).[16] This finding is similar to the results of our study in that lower concentrations of our extract did not have a significant adverse effect on normal cells and the adverse effect of the ideal concentration (1 mg/ml) on normal cells was only 2.8%, which was considerably low compared to the corresponding effect on cancer cells (killing off 45.1% of cancer cells). This highlights the importance of finding the optimal concentration of the extract for anti-cancer treatment, that is, the concentration that will maximize the cumulative anti-cancer effects of its constituents and various cellular mechanisms that they activate or suppress with minimal damage to normal cells.

Several studies have also reported that watercress supplementation can reduce DNA damage in lymphocytes and improve the blood anti-oxidant condition of healthy individuals, which means that this plant has a substantial potential to serve as a source of anti-cancer substances.[25-27]

In the end, it should be noted that as of the time of writing the article, the literature contains only two previous reports on the anti-cancer effect of watercress on oral SCC, which makes it difficult to discuss and interpret the findings without comparable data and references. Therefore, the field can benefit from further research, especially into the mechanism of the effect of watercress extract on cancer cells, to determine how this and other similar plants can be used in cancer treatments and particularly those for common oral cancers.

CONCLUSION

The aqueous extract of watercress affects the viability of cancer cells. This effect weakens with the increase in exposure time (inverse relationship) but strengthens with the increase in

the extract concentration (direct relationship). This extract also hurts the viability of healthy cells, but this effect only becomes noticeable at higher dosages. The best concentration of the extract was found to be 1 mg/ml. At this dosage, the extract managed to kill 45.1% of cancer cells (54.9% viability) and killed only 2.8% of normal cells (97.2% viability).

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Conflicts of interest

There are no conflicts of interest.

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