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ORIGINAL ARTICLE

Cytotoxic Effects of Aqueous Extract of *Portulaca oleracea* on Oral Cancer Cell Line

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ABSTRACT

Background: *Portulaca oleracea* as a herbal plant has been widely used in traditional medicine to cure many diseases. The therapeutic effects of this plant are exerted through its active ingredients with antioxidant properties. It has been shown that this plant may have cytotoxic effects on cancer cells. In the present study, cytotoxic effects of aqueous extract of *Portulaca oleracea* on oral cavity cancer cell KB cell line has been investigated.
Methods: KB cancer cell line was purchased from National Cell Bank of Pasteur Institute of Iran. Aqueous extract of *P. oleracea* was prepared by the maceration method. Cytotoxic effect of the aqueous extract of *P. oleracea* was assessed through different doses for 12, 24 and 48 hours using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Trypan blue. Also, morphological changes of the nucleus were carried out by Hoechst Staining.
Results: Rate of proliferation in cells treated with *P.olarecea* aqueous showed a significant decrease depending on dose and time; as with the increase of the dose and time, the more cells were decreased. There were also nuclear changes indicative of apoptosis in the cells treated with the extract.
Conclusion: It seems that *P.oleracea* aqueous extract has anti-proliferative effects on oral cavity KB cancer cell line. Further studies need to be undertaken to prove the efficacy and mechanism of its action.

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Introduction

Oral cancer is one of the most common types of cancer in the world and is regarded among the first ten causes of death due to malignancies. Epidemiological studies indicate that the prevalence of oral cancer varies in different countries of the world.[1] Surgey, chemotherapy and radiotherapy are treatment modalities used in advanced stages of the disease. All of these approaches are associated with various side effects. In spite of cancer prevention strategies and modern treatments and also different approaches for early diagnosis, herbal medicine can be suggested in parallel to synthetic drugs. It is noted that herbal medicine is considered as one of the main cancer treatment options in many countries.[2] Application of herbal medicine in cancer recently has drawn attention, so that various studies have been done to investigate antitumoral properties of local herbal medicines in different countries.[3] In this regard, researchers have reported more than 3000 plant species that can inhibit or prevent the growth of cancers.[4] Due to fewer side effects and having economical and longlasting effects, herbal medicine can be a desirable replacement or supplement for synthetic drugs.[5] Currently, researchers around the world are trying to find safe and more efficient treatments

for preventing and treating all types of cancers relying on herbal medicine. *P.oleracea* with the scientific name *Portulaca oleracea* L. from Purtulacaceae type is one of the valuable herbal medicines that grows wildly or planted in different parts of the world.[6] Based on traditional medicine, it can be used for the treatment of scurvy, persistent cough, cleansing, as antipyretic and efficient in healing burns, muscle relaxation and also cancer treatment.[7] It is composed of enameled materials, pectin, carbohydrates, protein, fatty acids, specifically Omega3 unsaturated fatty acids and Alkaloids. Also, it contains minerals such as iron, copper, potassium, selenium, and vitamins A, E, and C.[8] *P.oleracea* is abundant in antioxidants, including tocopherol, ascorbic acid, and glutathione.[9]

The present study has been carried out to investigate anti-cancer effects of *P.oleracea* in vitro. Further studies would be promising to find more efficient and newer anti-cancer drugs for cancer control.

Materials and Methods

Plant Collection

P.oleracea was collected from the herbal medicine garden in Hamadan at the end of spring in 2014. Detection of the plant was confirmed by the Basic Sciences, Faculty of Bu-Ali Sina University.

Extraction

Maceration method was implemented for preparing *P.oleracea* aqueous extract. Briefly, the dried plant was powdered using a cylindrical compression. About 20 grams of powder was added to100 ml of distilled water and within 72 hours it turned into a solution by a shaker.[10] Then, the extract was centrifuged by filter paper 10 rpm for ten minutes. It was concentrated at vacuum by Rotary and Baros Distillation and transferred to an oven of 40 °C until it was dried.[11] This extract was considered as the pure extract and stored in -20 °C.

Cell Culture

Oral Squamous Cell Carcinoma cell line (KB Cell line) was purchased from National Cell Bank of Iran (NCBI). The cells were cultured in RPMI-1640 containing 10% Fetus Bovine Serum (FBS), 100 IU/ml Penicillin and Streptomycin at 37 °C and 5% CO2 in an incubator.[12] Cell culture medium was changed every 3 days and the cells were subcultured at 70-80% confluency.

Treatment of the Cells with P.oleracea Extract

10^4 cells were cultured in 24 well plates and treated with 0 (as a control group), 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 µg/ml of aqueous extract for 12, 24 and 48 hours.

Cell Proliferation Assay Using MTT

The cells were cultured in 96 well plates and treated with different doses of aqueous extract of *P.oleracea*. After 12, 24 and 48 hours of culture, 20 µl of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) solution was added to each well and incubated for 3-4 hours. Then, the content of each well was replaced with 200 µl of DMSO solution and was shaken for 20 minutes. The optical density of each well was measured by ELISA reader at 570 nm. The test was repeated three times for each concentration and viability of the cells was calculated using the following formula:[13]

$$\text{Viability of cells} = \frac{\text{sample OD}}{\text{control OD}} \times 100$$

Cell Viability Test

Cytotoxicity effects of aqueous extract of *P.oleracea* on oral cancer cell lines were evaluated by Trypan blue staining. After 12, 24 and 48 hours culture of the cells with concentrations of 11, 12, 13, 14 and 15 µg/ml of the extract, trypan blue was added to the wells and after 3-5 minutes washed with Phosphate Buffered Saline (PBS). Blue stained cells were considered as dead cells and cell viability was reported as a percentage based on the below formula:[14]

Cell viability = living cells/total number of cells×100

Apoptosis Detection Using Hoechst Staining

Apoptosis was detected with evaluation of changes in the cell nucleus morphology using Hoechst Staining. Briefly, the cells were cultured in sterile slide within 6 well plates in 10^5 concentrations. Then, they were treated with 0, 11, 12 and 13 µg/ml extract for 12, 24 and 48 hours. Afterward, the cells were washed with PBS and fixed for one hour with % 4 Paraformaldehyde. The cells permeabilized with Triton-X 100 and washed with PBS for three times. The cells were stained with 4 µg/ml Hochest for 10-15 minutes in dark and visualized under fluorescence microscopy.[15]

Statistical Analysis

Statistical analysis was performed using software SPSS20. Multiple comparisons between more than two groups were performed by One-way ANOVA followed by Tukey test. P value less than 0.05 was determined at the level of significance among the groups.

Results

Aqueous Extract of P.oleracea Decreased Proliferation of Oral Cancer Cell Line

12 and 24-hour treatment of the cells with 11, 12, 13, 14 and 15 µg/ml concentrations of the extract showed significantly decreased cell proliferation compared to the control group (untreated) ($P<0.05$). The significant reduction of cell proliferation started at 9 µg/ml concentration of the extract after 48 hours ($P<0.05$) (Figure 1). As it is shown in figure1, increase in concentration and time resulted in more decrease in cell proliferation. The least proliferation rate was detected after 48 h in15 µg/ml concentration of the extract. Also, it is shown that more that 50% of the cells died after incubation with concentration of 12 µg/ml during 48 hours.

Cell Viability Decreased After Treatment of KB Cells with Aqueous Extract of P.oleracea

Cell viability significantly decreased after administration of 11, 12, 13, 14 and 15 µg/ml of extract of *P.oleracea* after 12, 24 and 48 hours ($P<0.05$). As it is shown in figure 2,

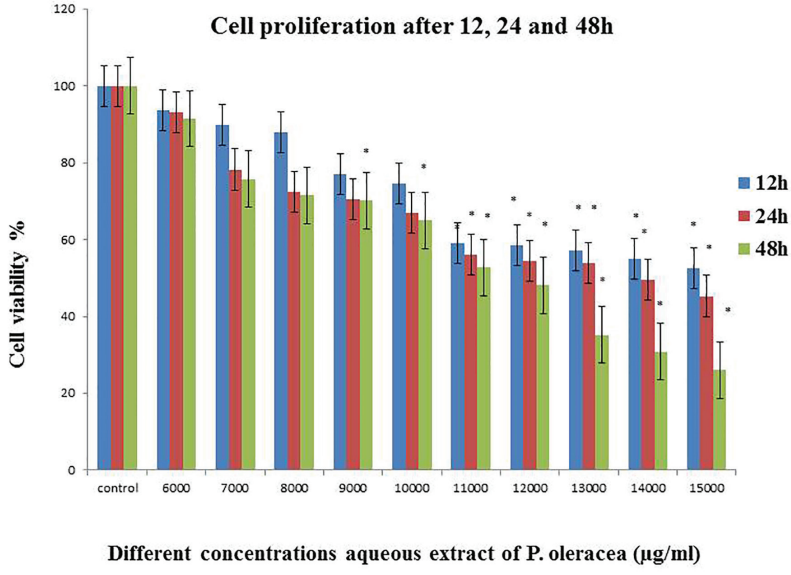

Figure 1: Proliferation rate of the cells after treatment with different concentrations of the aqueous extract after 12, 24 and 48 hours. It is clear that the extract inhibited cell growth in most doses, especially after 48h (P<0.05).

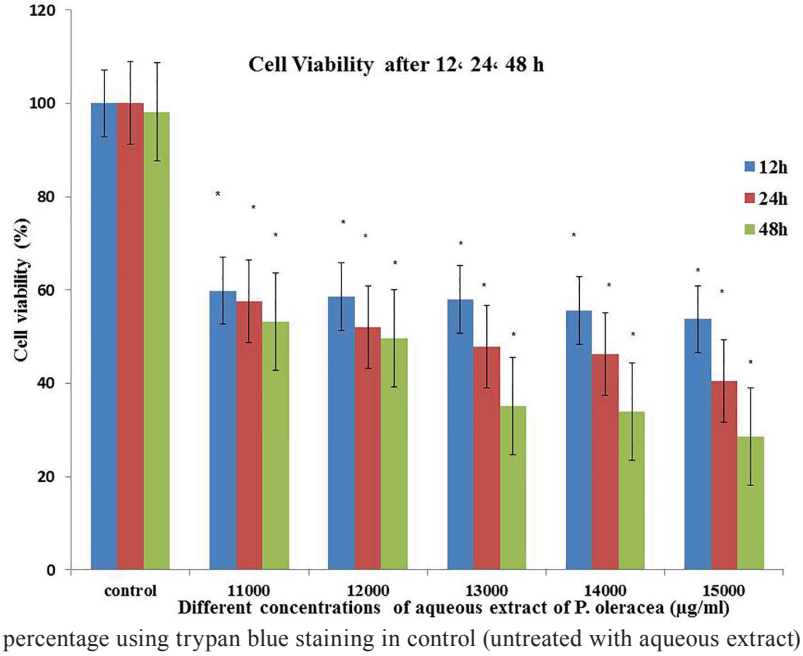

Figure 2: Cell viability percentage using trypan blue staining in control (untreated with aqueous extract) and groups received the extract. Cell viability significantly decreased after the cells were treated with the extract in all doses. The least cell viability was at 48 hours and 15000 µg/ml concentration (P<0.05).

the least viability rate was detected with concentration of 15 µg/ml after 48h. The highest rate of viability was recorded in the group with concentration of 11 µg/ml after 12 hours. It is shown that cytotoxicity of aqueous extract of *P.oleracea* is dose and time-dependent. As it is shown in figure 2, more than 50% of the cells died within concentrations of 12 and 13 µg/ml during 48 hours and 24 hours, respectively (P<0.05) (Figure 2 and 3).

Cell Nucleus Condensation Increased After Incubation with Aqueous Extract of P.oleracea

To know whether apoptosis had occurred after treatment with aqueous extract of *P.oleracea*in oral cancer cell line, Hoechst staining was performed. As it is indicated in figure 4, the cell nucleus was denser in the group received the extract rather than the control group (untreated well with 0 concentration of the extract). It was shown that after treatment with the extract, apoptosis was induced in the studied cells (Figure 4).

Discussion

Cancer is a major cause of death throughout the .world. According to the WHO, it is estimated that cancer is the cause of 83.2 million deaths during 2005-2015.[16]

Routine strategy for cancer treatment is that the active substance (ingredient) enters the body and influences cancer and the other cells. This causes consumption of high doses of chemotherapeutic drugs; furthermore, it causes damage to adjacent normal tissues of the body. In recent years, consumption of natural compounds to defeat cancer, has been suggested considering its fewer side-effects and promising effects.[17]

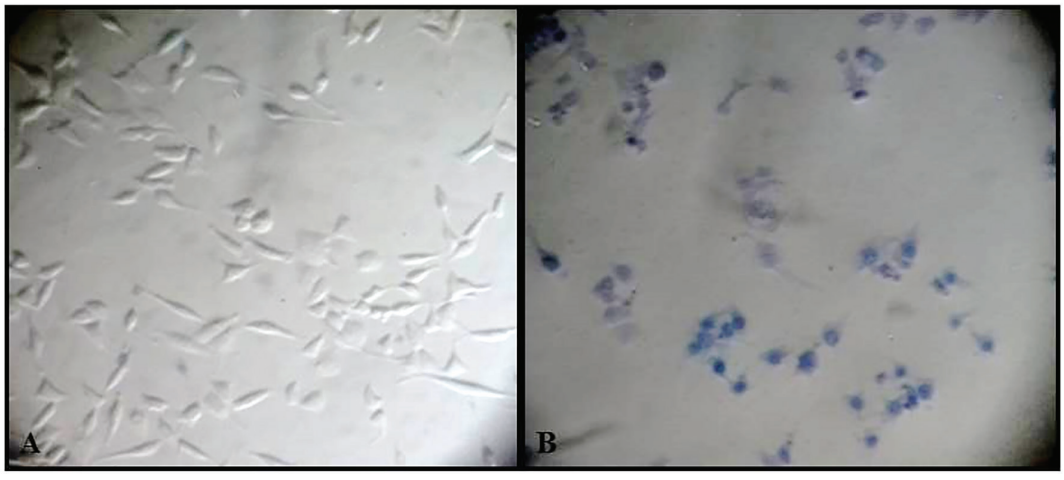

Figure 3: Trypan blue staining in control group (untreated with aqueous extract) (A) and experimental groups who received the extracts (B). The dead cells are shown in blue and most of them died after higher concentrations of the extract.

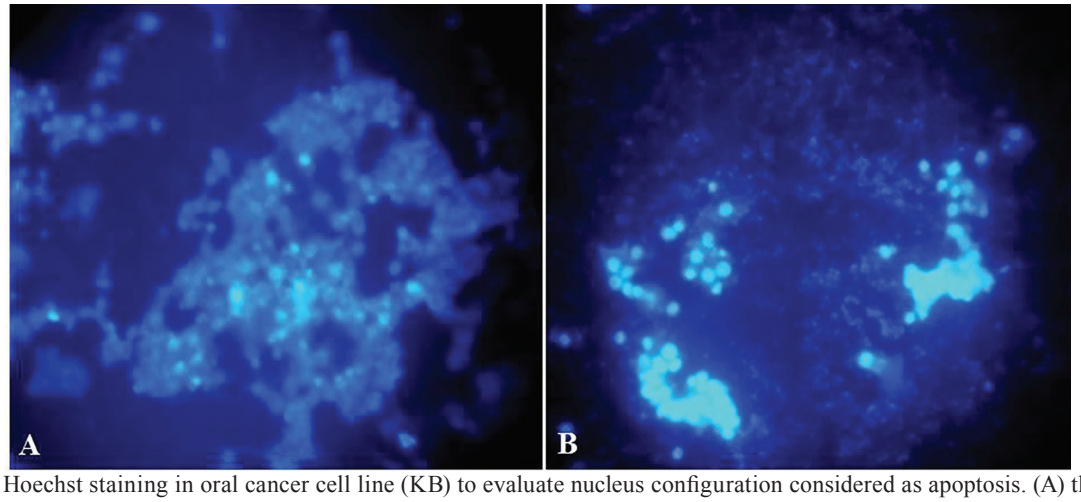

Figure 4: Hoechst staining in oral cancer cell line (KB) to evaluate nucleus configuration considered as apoptosis. (A) the control group (untreated with aqueous extract) with normal nuclear shape (B) the nucleus of cells treated with 11000 µg/ml of aqueous extract of P.oleracea showing deformation and condensation in nuclei.

On the other hand, regulation of apoptosis is one of the main concerns in the development of anti-cancer drugs. In recent years, many herbal compounds with various biologic effects have been introduced the modern science of pharmacy with the topic of treatment of different cancers. *P.oleracea* is rich in Alkaloids, flavonoids, phenolic acids and carbohydrates which are the basis for providing antibiotic, antioxidant, anti-mutagenic and anti-cancer properties with no harmful free radicals. Many studies have been carried out on therapeutic and anti-cancer effects of *P.oleracea*. Previous studies have shown that effective components of polyphenols obtained. from ethanol and polyphenol extract of *P.oleracea* have the ability to induce apoptosis in human colon cancer cells.[18] So far, no study has been performed on oral cancer cell line. Anti-cancer effects of ethanol and aqueous extracts of *P.oleracea* was demonstrated by exerting cytotoxicity on adenocarcinoma cancer cells of mouse breast through reduction of cell division and proliferation.[19]

Rua Zhao et al. indicated that polysaccharides in *P. oleracea* stopped cell cycle and induced apoptosis in cervical carcinoma cells that confirms its anti-tumor effects.[20] In another study conducted by Guo and colleagues, Portulacerebroside A resulted in chromatin condensation, nucleus fragmentation and increase in the percentage of apoptotic cells through activation of endogenous pathways or mitochondrial death pathway of apoptosis in HCCLM3 human liver cancer cells. This study showed that the compound isolated from *P.oleracea* increases the phosphorylation of proteins p38 MAPK and JNK and thereby it increases the permeability of the mitochondrial membrane, resulting in the release of cytochrome C and AIF from mitochondria to the cytosol. This leads to the activation of caspases 3 and 9 and thereby it activates endogenous apoptosis pathway.[21] It should be noted that inhibition of the growth and proliferation of the cells by inducing apoptotic internal pathway is dose and time-dependent.

In the present study, cytotoxic effects of aqueous extract of *Portulaca oleracea* on cancer cell line at different time points and concentrations were investigated. The results showed that the aqueous extract of the plant has the ability to reduce and inhibit the proliferation of KB cancer cells. It also inhibited cell division by apoptosis in cancer cells treated with aqueous extract of *P.oleracea* confirmed through Hoechst nuclear staining. The results of this study again show that the observed inhibition and reduction in cancer cells treated with aqueous extract of *P.oleracea* is dose and time-dependent.

Identification of active compounds in the extract of

P.oleracea and supplementary studies regarding its application in cancer therapy might be considered as a future strategy among scientists.

Conclusion

We showed that *portulaca oleracea* aqueous extract exerts anti-proliferation effects on oral cancer cell line in vitro and can be suggested to be used as a supplementary alternative therapy along with other cytotoxic chemotherapeutic drugs. Further studies need to be performed to prove its efficacy and find the mechanism of actions.

Conflict of Interest: None declared.

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