Research Paper: Cytotoxicity Effect of Hull-Less Seed Pumpkin Extract on Human Papillary Thyroid Cancer Cell Line

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ABSTRACT

Introduction: Thyroid cancer is the most prevalent endocrine malignancy tumor, and its incidence is increasing. Chemotherapy drugs like Paclitaxel (PTX) are a common treatment for cancer; however, they have many adverse effects. Plants are a source of anticancer agents. The present study assessed the cytotoxic effects of hydro-alcoholic extract in comparison with those of paclitaxel on Papillary Thyroid Cancer (PTC) cell line.

Methods: PTC cell line was treated by different concentrations of extract and paclitaxel for 24, 48, and 72 h. Cytotoxicity was examined through Trypan blue and clonogenic assays. Acridine Orange/Ethidium Bromide (AO/EB) staining was used for detecting apoptotic cells. The observations were statistically tabulated and analyzed.

Results: The Trypan blue staining results suggested that hydro-alcoholic extract had a cytotoxic effect on PTC cells. Our results of AO/EB staining revealed that the hydro-alcoholic extract of Hull-Less Seed Pumpkin (HLSP) in the concentration of ≥100 µl/mL induced significant apoptosis in the PTC cultured cell ($P<0.05$). In addition, the AO/EB staining data suggested an increase in the number of apoptotic cells with increasing the concentration of extract and paclitaxel. The clonogenic assay results indicated a decrease in colonies by increasing the concentration. Comparing the groups treated by paclitaxel or HLSP extract, with the control group revealed significant differences between them ($P<0.05$).

Conclusion: The HLSP extract had a cytotoxic effect on the human PTC cell line. Based on the adverse effects of chemotherapy drugs, this extract can be considered as a beneficial agent for PTC treatment.

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1. Introduction

Thyroid cancer is the most prevalent endocrine cancer, and the most common type of thyroid cancer is Papillary. Papillary Thyroid Cancer (PTC) comprises about 80% of such malignancies, and its incidence is rising in different populations [1, 2]. In Iran, thyroid cancer accounts for 1.8% of all cancers and 76.1% of endocrine cancers. Furthermore, PTC is the most frequent thyroid cancer type, with an incidence rate of 80%. Epidemiology studies in Iran also documented that the incidence of thyroid cancer in both genders is on the rise [3].

Current chemotherapy drugs, such as doxorubicin, daunorubicin, Paclitaxel (PTX), docetaxel, and cyclophosphamide are used as the first-line treatment for many cancers; however, these drugs have adverse effects, like alopecia and Chemotherapy-Induced Neuropathic Pain (CINP) [4-6]. Paclitaxel, one of the most important members of the taxon family, is a well-known anticancer drug used for treating various cancers [7]. It exerts its cytotoxic effect by arresting mitosis through stabilizing microtubules by preventing their depolymerization [8]. The available chemotherapy drugs are unbeneficial to all cases and have serious adverse effects on human health; thus, finding new anticancer agents with fewer side effects seems imperative. Studies indicated that herbal medicines with anticancer potential are considered as promising therapeutic agents.

Moreover, different plants have anticancer properties [9, 10]. Cucurbita pepo L. (Cucurbitaceae), commonly known as the "pumpkin" seed, has extensive therapeutic properties, including antibacterial, antiviral, anti-inflammatory, analgesic, anti-mutagenic, and anticancer effects. Furthermore, pumpkin seed improves Benign Prostate Hyperplasia (BPH) associated symptoms [11, 12].

Seeds and seed oil of pumpkin are rich in proteins, fithostrol, fatty and non-saturated fatty acids, including linoleic, linolenic, palmitic, stearic, vitamins (A and E), phenol antioxidant compounds, such as carotenoids, lutein, tocopherol, Gama, chlorophyll, as well as elements, like zinc and selenium [11, 13]. In addition, a diet containing high amounts of pumpkin seeds reduced the risk of gastric, breast, lung, and colon cancer [12]. Moreover, different carotenoid pigments in pumpkin seed oil prevent prostate cancer [12]. In a research study, pumpkin extract reduced the weight of s-180 tumors in mice [14]. Also, several base proteins, called MAP2 (MW 2249 Da) and MAP4 (MW 4650 Da) were extracted from pumpkin seed, which has inhibitory effects on the blood cancer of K_562 cells. In addition, there exist other proteins in pumpkin seeds, which limit the proliferation of melanoma [12, 15]. In this study, the anticancer effects of hydro-alcoholic extract of HLSP were examined and compared with those of paclitaxel against human PTC cells in the lab.

2. Materials and Methods

Hull-less seed pumpkin (Cucurbita pepo subsp. pepo var. Styriaka) and human PTC cell line were obtained from VBG Company (Rasht, Iran) and Pasteur Institute of Iran, respectively. All procedures were approved by the Ethics Committee of Guilan University of Medical Sciences.

The PTC cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 100U/mL penicillin (Sigma-Aldrich), and 100µg/mL streptomycin (Sigma-Aldrich). Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed twice a week [16]. The frequency(%) of dead cells was determined by Trypan blue staining [16].

For the preparation of hydro-alcoholic extract, the method described by Navneet Kumar Yadav et al. was followed [17]. Briefly, a measured quantity of 50g of powdered and dried seeds of pumpkin was chopped and soaked in 500mL of ethanol (80%v/v) for 72 h. The suspension was then passed through filter paper and concentrated using a rotary vacuum evaporator at 40°C. Specific concentrations of hydro-alcoholic extracts (1, 20, 50, 100, 200, 800, 1600, 2400, 2800, 6400, and 100000 µg/mL) were prepared with phosphate buffer (pH=7.4). The hydro-alcoholic extracts were sterilized by 0.22 µm microbiological filters and maintained at 4°C before use.

To evaluate the effects of the drug on cells' viability, Trypan blue staining was used. To conduct Trypan blue staining, after performing the third passage and cell counting by Neubauer slide, the equal number of 80000 cells were added to any six-cell well plate. Then, by adding complete media, the volume of all wells was increased to 2 mL. After 24 h, all wells were first observed and inspected by an inverted microscope for proper growth and the unpollutedness of cells. Next, the prepared concentrations from the hydro-alcoholic extract of HLSP and paclitaxel were separately added to each well. To observe and investigate cell growth inhibition, Trypan blue staining was performed after 24, 48, and 72 h. For the preparation of this stain, 4.0% of Trypan blue powder (Sigma-Aldrich) was added to the sodium phosphate buffer (pH=7.4). To stain wells, each well was initially passaged separately and centrifuged, respectively. Then, 20 µL of the cell suspension was mixed with 20 µL of Trypan blue staining solution, and 10 µL of the resulting mixture was put on Neubauer slide. In this staining, the color of cells with defect membranes was observed as blue, and live cells were observed as completely apparent and colorless. Experiments were performed three times in triplicate.

To detect apoptotic cells, PTC cells were seeded in 96-well plates at a density of 5×10³ cells per well and incubated for 24 h; then, treated by different concentrations of paclitaxel (0.00001-6000 µg/mL) or extract (1-100000µg/mL) for 24, 48, and 72 h. To show the cell death, suspensions from plate cells were prepared and stained after 24, 48, and 72 h. As described by Aline Monezi Montel et al. [18], 50 µL of Acridine Orange/Ethidium Bromide (AO/EB) dye (a mixture of AO (100 µg/mL) (Sigma) and EB (100 µg/ml) in PBS) (Sigma-Aldrich)) was prepared. Cells were observed and counted under an inverted fluorescence microscope (Olympus, IX71, Japan). This experiment was preformed three times in triplicate. AO is absorbed both by living and dead cells. AO enters the cell nucleus and offers a green view of living cell chromatin; however, EB only penetrates the dead cells and gives an orange view to the chromatin of dead cells under the fluorescence microscope.

To distinguish the cell viability after treatment by paclitaxel and the extract, clonogenic assay was performed as described by Jinichi Mori et al. [19]. Briefly, 2 mL of DMEM, containing 2000 cells, was added to six-well plates. They were incubated at 37°C for 24 h. After incubation time, the cells were exposed to different concentrations of paclitaxel (0.00001-20 µg/mL) or the extract (200-6400 µg/mL) for 7 days. After 7 days, the cells were stained by Giemsa dye, as described above. The cell colony images were prepared, and more than 50 cell colonies were counted using a light microscope.

The obtained data were analyzed by SPSS. Statistical comparisons between the study groups were made with one-way Analysis of Variance (ANOVA) and Tukey test. Mean differences were considered significant at P<0.05.

3. Results

According to the results obtained from Trypan blue staining, in terms of the concentration of 1mg/mL of hydro-alcoholic extract, there were no significant differences in the controls between the three examination times (24, 48, and 72 h). However, in the concentration of 200µg/mL, the number of dead cells increased, and the viability of cells declined up to 15%. In the concentration of 800 µg/mL, the viability of cells declined up to 30%, as well. In these concentrations, time had no significant impact on the increase or decrease in cell death. In the concentration of 1600 µg/mL, cell viability was extensively reduced. Moreover, in the concentration of 6400 µg/mL, the viability of cells reached the minimum, and the number of dead cells increased significantly ($P \leq 0.05$). In the paclitaxel treated groups, in the concentration of 0.00001 µg/mL, except for the case of 72 h, cell death was not observed; however, with an increase in concentration, a significant reduction occurred in cell viability ($P \leq 0.05$) (Figure 1).

To illustrate the frequency (%) of apoptotic cells, after treating PTC cells by different concentrations of paclitaxel and the HLSP extract, AO/EB staining was carried out. In the AO/EB staining, live cells are specified with green color, and dead cells are specified with orange to red color (Figure 2). To determine the amount of cell growth

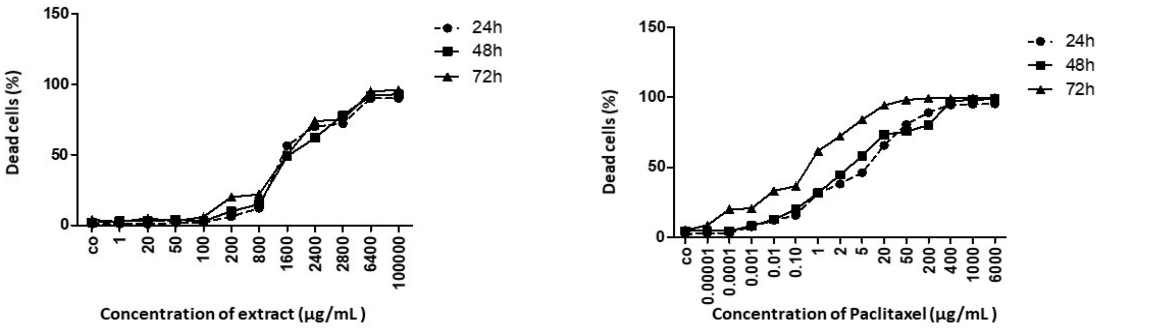

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Figure 1. Comparing the frequency (%) of dead cells in PTC cells treated by HLSP extract and paclitaxel at 24, 48, and 72 h

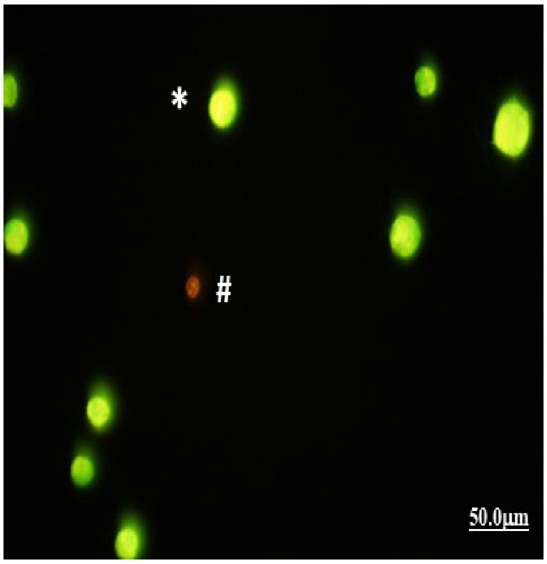

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Figure 2. AO/EB staining
*: Indicates live cells; #: Indicates dead cells

and colony forming PTC cells after treatment with the extract, clonogenic assay was used.

Similar to the results of Trypan blue staining and AO/EB staining, results of clonogenic assay showed when the concentration of Paclitaxel or pumpkin hydro-alcoholic extract increased, a significant decrease in the number of colonies was observed in comparison with the control group. The percentage of colonies formed after a week was 47.91% in the concentration of 1600 in the extract compared to 58.75% in the concentration of 0.01 in Paclitaxel (compared to the control group) ($P<0.001$) (Figure 3 and Figure 4).

4. Discussion

The present study investigated the pharmacological effects of HLSP on the PTC cell line. Due to the presence of some crucial components, such as sterols, polysaccharides, fixed oils, para-aminobenzoic acid, proteins, and peptides, pumpkin has been considered as a valuable health nutrient. Pumpkin seeds are a useful source of protein and essential amino acids, fatty acid, linoleic acid, and some beneficial ions, such as K, Cr, Na, Mg, Zn, Cu, Mo, and Se [20, 21]. Various extracts and fractions obtained from pumpkin have robust antioxidant activity and profoundly affect the prevention and treatment of some endocrine and vascular diseases [22, 23]. Pumpkin seed and its associated oil contain a rich source of tocopherol (vitamin E) (as an essential antioxidant) [24]. In addition, administrated pumpkin extract increased the serous and hepatic activity of Superoxide Dismutase (SOD) and decreased the lipid peroxidation of cell membranes [12]. Furthermore, pumpkin extract could increase the SOD and decrease the malonaldehyde content in tumor-containing mice serum [25].

In the thyroid gland, epithelial cells create some reactive oxygen species, i.e. required for the synthesis of T3 and T4 hormones. Nevertheless, when the aforementioned cells produce excessive ROS, they cause toxic effects on thyroid cells [26, 27]. Among environmental factors causing or predisposing to thyroid cancer, oxidative stress plays an important role. Oxidative stress is a biochemical condition characterized by the accumulation of ROSs, such as superoxide anion radical, hydroxyl radical, hydrogen peroxide, and imbalance between pro-oxidant and antioxidant compounds [28]. Wang et al. investigated the total oxidant/antioxidant status in the sera of patients with thyroid cancers. They suggested that ox-

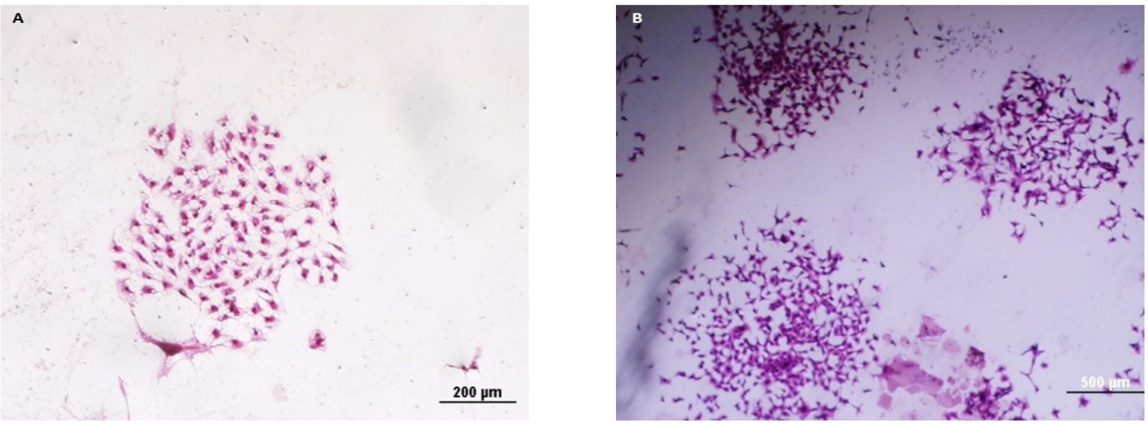

Figure 3. Colonies formed by PTC cells after treatment
A. HLSP extract; and B. Paclitaxel

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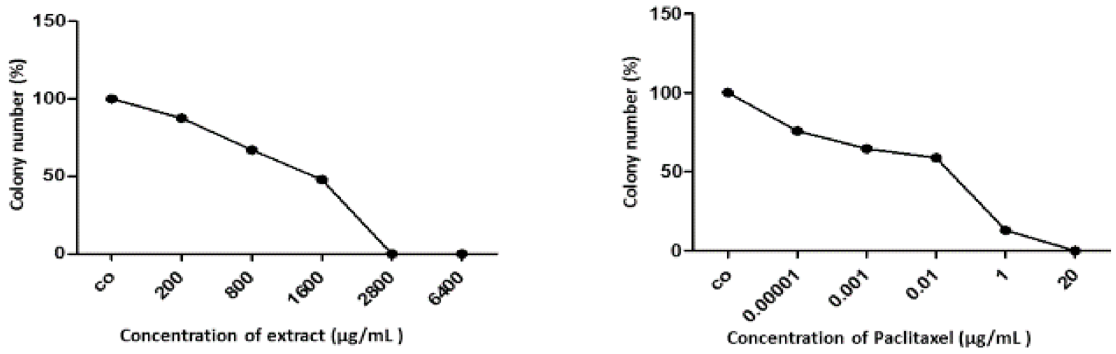

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Figure 4. The effect of different concentrations of HLSP extract and paclitaxel on PTC cells colony formation

idant parameters increased, and antioxidant parameters decreased in thyroid cancer [29]. Muzza et al. surveyed the oxidative stress and the subcellular localization of the telomerase reverse transcriptase in PTC. Their data demonstrated that the intracellular H2O2 (as oxidant parameters) is significantly higher in PTCs than in healthy thyroid tissues [30]. Furthermore, they concluded that mitochondrial oxidative stress was not markedly different in normal and tumor tissues [30]. Given the crucial role of oxidative stress in the pathogenesis of PTC and the proven antioxidant activity of pumpkin seeds, selecting pumpkin seed in the present study was precious.

Programmed cell death, or apoptosis, is a mechanism by which cells undergo death to regulate cell propagation or in reply to genomic damage. This study surveyed the anticancer activity of HLSP on the PTC cell line employing apoptosis. Understanding apoptosis has provided a critical basis for cancer therapies that can induce tumor cell death or sensitize them to chemotherapy and radiation therapy [31-33]. In the aforementioned novel method of cancer therapy, some agents target the extrinsic pathway of apoptosis, like tumor necrosis factor-related to apoptosis, and some agents target the intrinsic Bcl-2 family pathway [31].

In the present study, AO/EB staining was used to depict nuclear changes and apoptotic body formation as a typical characteristic of apoptosis. To avoid the misidentification of live and dead cells, special attention was paid to some key features of these cells. Both live and dead cells were stained with AO dye; however, EB stained were the only cells that lost cell membrane integration. In this staining, live cells would appear in green. Early apoptotic cells are apparent green accompanied by nuclear bright dots as a consequence of genomic condensation and nuclear fragmentation process. Late apoptotic cells are apparent orange (due to interaction with EB dye) [34]. AO/EB staining results suggested that the hydro-alcoholic extract of HLSP in the concentration of ≥100μl/mL induced significant apoptosis in the PTC cultured cell ($P<0.05$). In addition to the aforementioned staining, we performed clonogenic assay to ensure the existence of apoptosis. The data obtained from clonogenic assay are consistent with those of AO/EB staining. Additionally, based on our perceptual analysis, the number of apoptotic cells in the treatment group with concentrations of 200, 800, 2400, and 2800 μl/mL were more than that of the controls.

According to the literature, the present study was the first to investigate the effects of HLSP on thyroid cancer. Previously, pumpkin therapeutic effects on breast, blood, and prostate cancer have been proven. Richter et al. investigated the phytoestrogen extracts isolated from pumpkin seeds on breast cancer. Their results highlighted a potential role of pumpkin seeds in breast cancer prevention and treatment [35]. As previously mentioned, the inhibitory action of pumpkin is also proven on leukemia and prostate cancer.

5. Conclusion

Hydro-alcoholic extract of HLSP induced PTC cell apoptosis in vitro. The results, as mentioned above, could highlight the potential role of HLSP in PTC prevention and treatment. However, for making precise decisions about the effect of HLSP on PTC, more molecular investigations are required.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Guilan University of Medical Sciences (Code: ???).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflicts of interest

There are no conflicts of interest to be declared.

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