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ARAŞTIRMA / RESEARCH

Haplophyllum buxbaumii ekstresinin skuamöz hücreli karsinoma üzerine apoptotik etkileri

Apoptotoic effects of Haplophyllum buxbaumii extract on squamous cell cancer

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Abstract

Purpose: Oral cancers are malignant neoplasms affecting the structures or tissues in the mouth and oral squamous carcinoma is the most common neoplasm of the skin, lips and oral cavity mucous membrane. It has been suggested that Haplophyllumbuxbaumii, which is a perennial herbaceous plant, has antioxidant activity. The aim of this in vitro study is to investigate the anticancer effects of Haplophyllum on oral squamous cancer cell lines.
Materials and Methods: The effects of Haplophyllumbuxbaumii plant extract on squamous cell carcinoma were evaluated by Cell viability Assay, Total Protein Quantification and by activity of cleaved caspase-3, Bax and Bcl-2 determined by ELISA method.
Results: The results of the tests showed that Haplophyllum extract application reduced the viability of squamous cell carcinoma cells while activating the apoptotic pathway when compared to the controls.
Conclusion: The findings of the study suggest that Haplophyllumbuxbaumii plant extract may have some anticancer potential to contribute to the studies on squamous cell carcinoma therapy.

Keywords:. Haplophyllum buxbaumii, squamous cell cancer, apoptosis

Öz

Amaç: Ağız kanserleri, ağızdaki yapıları veya dokuları etkileyen malign bir neoplazmdır. Oral skuamoz hücreli karsinoma deri, dudaklar ve oral kavitede mukoz membranda en sık görülen neoplazmdır. Haplophyllum buxbaumii, çok yıllık otsu bir bitkidir ve antioksidan özellikleri olduğu bildirilmiştir. Çalışmanın amacı Haplophyllum'un oral skuamoz kanser hücreleri üzerindeki antikanser etkilerini değerlendirmektir.
Gereç ve Yöntem: Haplophyllum buxbaumii bitkisinden elde edilen ekstrenin skuamöz hücreli karsinom hücreleri üzerine etkileri, hücre canlılık testleri, total protein sayımı ve Elisa yöntemiyle caspase-3, Baxve Bcl-2 aktivitesi ile incelenmiştir.
Bulgular: Çalışmanın sonuçlarına göre Haplophyllum ekstresi uygulaması, kontrollerle karşılaştırıldığında skuamöz hücreli karsinom hücrelerinin canlılığını azaltırken apoptotic yolağı da aktive etmiştir.
Sonuç: Haplophyllumbuxbaumii bitki ekstresini skuamöz hücreli karsinom hücreleri üzerinde toksik etki göstermiş olup bu kanserin terapisine yönelik çalışmalara katkı sağlayacaktır. Çalışmamız bu bitkinin antikanser potansiyeli olduğunu göstermektedir.
Anahtar kelimeler: Haplophyllum buxbaumii, skuamöz karsinoma, apoptozis

INTRODUCTION

Oral cancers are malignant neoplasms that affect the structures or tissues in the mouth. These cancers may be primary lesions that originate from the mouth or metastases from distant areas or extending from adjacent structures presenting as lesions affecting the oral tissues. Oral cancer constitutes 2-4% of all diagnosed cancers, and 5,000 new cases are added to the annual cases of oral cancer[1, 2]. Squamous epithelium is the primary surface structure of the skin, lips and oral cavity mucous membrane. 86-95% of malignant tumors of the head and neck region originate from this structure. Oral squamous cell carcinoma, which accounts for more than 90% of oral cancers, may affect the mucous membrane of the mouth and oropharynx[3]. Generally, it is a malignant tumor that starts with dysplasia in the

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multi-layered flat epithelium lining the oral cavity and develops with the invasion of the neoplastic cells into the subepithelial area through the basement membrane. The first symptoms are painless ulcer and/or ulcerated exophytic lesion on the mucousa[4].

Many studies have proven that most chemotherapeutic drugs are cytotoxic and target non-specific high proliferative cells. This then leads to the poor prognosis and lower quality of life in cancer patients. In this context, novel anticancer agents that selectively target cancer cells are continuously investigated[5].Recently, there has been an increasing tendency to natural sources using plants as alternative treatment methods for various forms of diseases.

Haplophyllumbuxbaumii (HP) is a perennial herbaceous plant which can be grown in steppe, barren and fallow land or cultivated land. The general distribution of the HP plant is the Syrian desert and Iran. It can also be found around Şanlıurfa in Turkey. Several studies have shown that subspecies of Haplophyllum is protective against oxidative stress[6]. Although the protective effects of this plant (and its subspecies) on normal cells have been reported; to our knowledge, this is the first study to analyze the anticancer effects of this plant

The aim of this in vitro study is to analyze the apoptotoic effects of HP on oral squamous cell culture.

MATERIALS AND METHODS

This in vitro study has been performed in Cukurova University, Faculty of Medicine, Department of Pharmacology. As certified cell cultures were used, no ethics committee approval was needed. The above-ground parts of the HP plant were used in this study. HP was dried in the oven; powdered and thoroughly homogenized for 15 minutes with an ultrasonicizer with methanol: water solution (prepared in a ratio of 500 g (1: 1)) and then incubated in shaker at 40 ° C overnight. Alcohol solvents were blown with a rotary evaporator not exceeding 40C, and the preparate was filtered with whatman. Finally the extracts were obtained by powdering in the lyophilizer.

Cell culture

Human SCC-25cell line was obtained from American Type Culture Collection (ATCC). squamous cancer cells were grown on dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate and supplemented with 400 ng/ml hydrocortisone and 10% fetal bovine serum. The cells were incubated in a humidified atmosphere at 37°C in 5% CO_2.

Cell homogenization

Cells (5×10^4 cells/cm^2) were exposed to 20µg/ml HP extract for 48 h. Cells were then washed in PBS and lysed in RIPA buffer (150mmol/L NaCl, 0.5%, TritonX-100, 20mmol/L EGTA, 1mmol/L dithiothreitol, 25mmol/L NaF, 50mmol/L Tris–HCl [pH 7.4], 1mmol/L Na3VO4) for 15 min on ice followed by centrifugation at 15000 rpm for 20 min and supernatants were taken and pellets were discarded.,

Cell viability assay

Cell viability of SCC-25 were analyzed by MTT[7]. 5 mg/mL dose of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) were prepared by dissolving in filtered phosphate-buffered saline (PBS). Cells were plated at a density of 104 cells/cm^2 in 96-well plates in a final volume of 180 µL of medium and were incubated overnight. The cells were then treated once with dose of %2-40µg/ml of HP and were examined after 48h. After completion of the treatment with HP, MTT was added to each well at a 1/10 volume for 3 h at 37°C. The supernatants were carefully aspirated, 100 µL of dimethyl sulfoxide was added to each well, and the plates were agitated to dissolve the crystal product. Absorbance of plates were measured at 570 nm Quantitative Analysis.

Total protein quantification

Bradford method was used to quantify the total protein in homogenized cells. By using Bovine serum albumin (1µg/ml), 1, 2, 3, 5, 7, 8, 10 (µg/ml) standards are prepared. 10 µl is taken from every sample and completed to 100 µl by adding distilled water. 1 ml Bradford solution is added to standards and samples, vortexed and absorbance at 595 nanometer are measured manually. Protein quantification (µg/µl) is done according to the standard curve drawn in Prism software[7].

ELISA Test

ELISA (Enzyme Linked Immunosorbent Assay) test

was used to examine the expression and activity of cleaved caspase-3, Bax and Bcl-2 as previously described[8, 9]. 100µl of homogenates were added to Elisa plates which were then covered with aluminum and incubated for 2h at 37ºC. Then 100 µl of reactive A solution was added and again incubated for 1h at 37ºC. The plates were then buffered with solution and 100 µl of reactive B solution was added to the plates and incubated for 1h 37ºC'. The plates were buffered again with solution for 5 times and 90 µl of substrate solution was added were incubated again for 30 minutes. Finally 50 µl of stop solution was added and the optical densities of the plates were evaluated by microplate reader at 450 nm to determine the amount of cleaved caspase-3, Bax and Bcl-2 enzymes.

Statistical analysis

Parameters of control and HP-treated groups were compared by unpaired Student's t test. Data is expressed as means ± SD. Difference at $p < 0.05$ level was considered as significant.

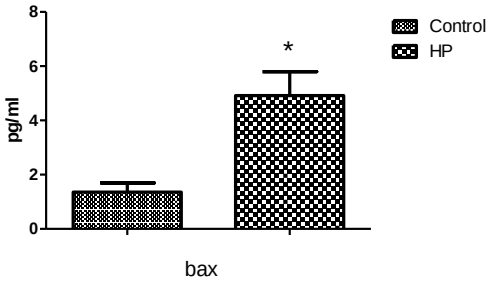

Figure 1. Effect of HP treatment on bax expression.
The data are presented as mean ± SEM. Differences between parameters of control and extract treated group were analyzed unpaired Student's t test. *difference between control and HP treated group is significant with $p < 0.05$.

RESULTS

In this study, SCC-25 cells were exposed to a total extract of HP at a concentration of 20 µg/ml for 48 hrs. The control group was consisted of untreated SCC-25 cells. The results have shown that HP treatment significantly raised levels of the Bax (4.917±0.8796 for HP vs. 1.354±0.,3406 for controls) (Fig 1) and cleaved-caspase 3 (5.186±1.107 for HP vs. 1.371±0.1782 for controls (fig 2) in SCC-25 cells when compared with the control group ($p<0.05$).

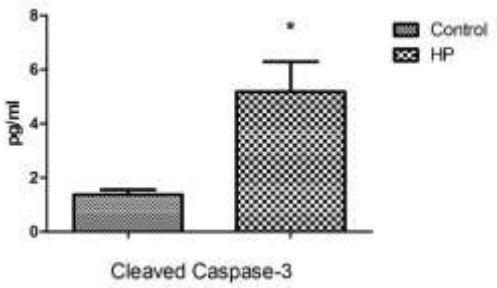

Figure 2. Effect of HP treatment on caspase-3 activity.
The data are presented as mean ± SEM. Differences between parameters of control and extract treated group were analyzed unpaired Student's t test. *difference between control and HP treated group is significant with $p < 0.05$.

On the other hand, HP treatment significantly reduced levels of the anti-apoptotic Bcl-2 (1.363±0.2283 for HP vs. 3.811±0.2536 for controls) (fig 3) in SCC-25 cells when compared with the control group ($p<0.05$). 48 hour HP treatment also reduced cell viability SCC-25 cell in various doses (fig 4, table I).

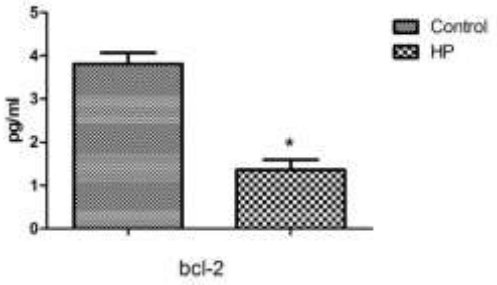

Figure 3. Effect of HP treatment on bcl-2 expression.
The data are presented as mean ± SEM. Differences between parameters of control and extract treated group were analyzed unpaired Student's t test. *difference between control and HP treated group is significant with $p < 0.05$.

Table 1. Means and standard error of MTT

	Control	5□ HP	10□ HP	20□ HP	40□ HP
Mean	103.8	96.29	84.00	48.60	16.80
Standard Error	1.744	1.248	2.017	2.731	2.888

HP: Haplophyllum buxbaumii

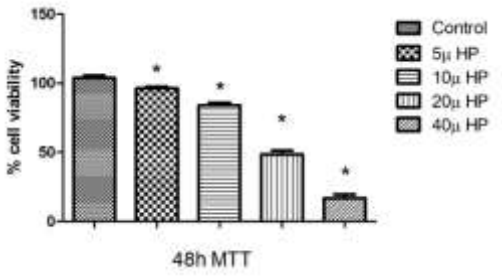

Figure 4. Effect of extract treatment on cell viability (MTT).

The data are presented as mean ± SEM. Differences between parameters of control and extract treated group were analyzed unpaired Student's t test. *difference between control and HP treated group is significant with $p < 0.05$.

DISCUSSION

There is limited information on the medical use and anticancer effects of Haplophyllumbuxbaumii in the literature. The results of the current study showed that the HP plant can reduce the viability of SCC-25 cancer cells at various doses via inducing the apoptotic pathway.

In recent years, there has been a significant increase in the interest in naturally acquired compounds in cancer treatment. The properties of plants used as food, vegetables, fruits or spices, which are rich sources of bio-nutrients or bioactive phytochemicals in natural substances, have attracted attention. However, detailed studies are needed to find out the biological safety of these compounds when used medically. Plants and their derivatives have been used in medical treatments since ancient times. Various plants with many different cultural origins are used by physicians to cure many diseases. Studies conducted in recent years present as an option for cancer prevention and treatment of phytochemicals in plants[10]. The use of plant-derived products is non-toxic to normal cells and is better tolerated. For this reason, it attracts the attention of modern drug discovery[11]. Some plant-based compounds presented effects on carcinogenic activity in a variety of pathways[12].

The estimated figures explored revealed that the plant population contains at least 250,000 species and only 10 percent are researched for pharmacological applications. Phytochemicals and their derivatives in roots, leaves, flowers, stems and bark perform various pharmacological functions in human metabolic systems[10, 13, 14]. As a result of newly applied drug production and innovation studies, the interest in phytotherapy drugs with less side effects compared to chemotherapy has increased. The biggest disadvantage of the drugs used in cancer treatment is that they are not specific and their side effects are quite high. Therefore, it is impossible to talk about the application of the radical treatment method.

The results of the current study has shown that HP application has increased apoptotic mediators while decreasing antiapoptotoic mediators. In addition the MTT test has shown that that the cell viability has decreased by the activation of apoptotic pathway. These results suggest that HP has toxicity on cancer cells while protecting the normal ones. Previous studies on HP have shown that its essential oils have potentially protective effects on oxidative stress, highly cleaning efficacy and protective effects on human stroma U373-MG cells against H2O2 damage [15, 16]. It has been proven that essential oils inhibit ROS production caused by H2O2. In a study comparing antioxidant activity of polyphenols and HP alcholoids, it has been shown that although polyphenols have higher antioxidant capacity, HP alcholoids have also strong capacity. Similarly, other studies have reported that the ethonolic extract of HP had higher (98%) antioxidant capacity compared to Vitamin E and the glutation levels in diabetic rats have been restored by application of essential oils[15, 17].

The main limitation of this study is the use of single cell line. Therefore, further studies on the effects of HP on other cancer lines and molecular pathways are needed to evaluate the exact anticancer effects of HP.

The current study is pioneering as HP plant extract is tested on squamous cell cancer for the first time. The results show that this plant may have anticancer activity. Further in vitro and in vivo studies are needed to confirm the effects of HP plant in the treatment of squamous cell cancer.

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