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Research Article
Haplophyllum buxbaumii Induces Apoptosis via MAPK and AKT/mTOR Pathways in MCF-7 Breast Cancer Cell Line

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Abstract
Background and Objective: Breast cancer is the most common type of cancer in women. Recently, studies on breast cancer treatment by plant-based extracts have increased. *Haplophyllum buxbaumii* (HPL) is a perennial herbaceous plant. The HPL has antioxidants and anticancer effects. Hence, this study aimed to show the apoptotic effect of HPL and its effect on the AKT/mTOR and MAPK pathway in breast cancer. **Materials and Methods:** The MCF-7 cells were cultured and treated with HPL in various doses. The cytotoxic effect of HPL was evaluated by the MTT method. In addition, mediators of apoptotic, AKT/mTOR and MAPK pathways were analyzed by the ELISA method. The data were analyzed using the Student's t-test. **Results:** The HPL significantly increased expression of the proapoptotic protein, Bax and cleaved-caspase 3, in MCF-7 cells. The HPL treatment also significantly reduced the expression of Bcl-2 protein which is anti-apoptotic in MCF-7 cells. The HPL also decreased the viability of MCF-7 cells by dose-dependent. The viability test also showed that the cells entered the apoptosis process. The HPL treatment decreased the activity of p-ERK, p-JNK, p-AKT and p-mTOR in MCF-7 cells. **Conclusion:** The HPL induces apoptosis and inhibits AKT/mTOR and MAPK pathways in breast cancer. This study revealed that HPL can be used as a potential anticancer agent in breast cancer treatment as it induces the antiproliferative and apoptotic effects of HPL on human breast cancer cell MCF-7.

Key words: *Haplophyllum buxbaumii*, breast cancer, MCF-7, apoptosis, AKT/mTOR, MAPK

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Breast cancer is the most common type of cancer in women and accounts for 30% of cancers in women. The 15% of cancer-related deaths in women are due to breast cancer[1]. The focus is on alternative treatments because of the presence of many mutations in breast cancer, epigenetic and transcriptional changes, resistance to drug treatments and individual differences in response to treatment[2]. In addition to traditional treatments such as chemotherapy and radiotherapy, breast cancer vaccines, AKT/mTOR inhibitors, Histone Deacetylase (HDAC) inhibitors, JAK/STAT inhibitors, receptor tyrosine kinase inhibitors, androgen receptor inhibitors, Poly ADP Ribose Polymerase (PARP) inhibitors, steroid sulfatase inhibitors, farnesyl transferase inhibitors (FTI) are among the current treatments. These current treatment approaches are based on the inhibition of signaling pathways known to play an important role in breast cancer[3-6].

The MAPK signaling pathway consists of RAF, MEK and ERK proteins, which are essential for proliferation in many cancer cells as well as in normal cells. Both ERK and JNK MAPK pathways can be activated by RAS proteins. The AKT/mTOR and MAPK pathways are one of the most activated pathways in breast cancer and control many processes such as proliferation, invasion, metastasis and chemotherapy resistance. Inhibitors targeting these pathways are generally insufficient to suppress tumor growth due to the cross-interaction between these pathways[7].

Haplophyllum buxbaumii is a perennial herbaceous plant. Its general distribution is the Syrian desert and Iran. The HPL, which can grow in steppe, barren and fallow land or cultivated lands, is a plant that can reach an altitude of 1400 m and can find extreme living opportunities[8]. It is seen in Şanlıurfa in Turkey. Recent studies have shown that this plant has anticancer properties[9]. The number of studies with this plant is limited. Therefore, this study was planned to investigate the apoptotic effects of HPL on breast cancer and its effects on the PI3K/AKT/mTOR signaling pathway.

MATERIALS AND METHODS

Study area: This *in vitro* study was performed at Cukurova University, Faculty of Medicine, Department of Pharmacology in May, 2022.

Plant material and extraction: The HP was collected at flowering time in May, 2022 from Şanlıurfa. The above-ground parts (leaves, stems and flowers) of the HP plant were used in this study. The HP was dried in the oven, powdered and thoroughly homogenized for 15 min with an ultrasonicizer with methanol:water solution (prepared in a ratio of 500 g (1:1)) and then incubated in a shaker at 40°C overnight. Alcohol solvents were blown with a rotary evaporator not exceeding 40°C and the preparation was filtered with Whatman paper. Finally, the extracts were obtained by powdering in the lyophilizer (TRS-2-2, Teknosem Inc, Istanbul, Turkey).

Cell culture: Human breast cancer cell line MCF-7 was purchased commercially from the American Type Culture Collection (ATCC). The MCF-7 cells were added to 25 cm^2 flasks that contain DMEM (Dulbecco's Modified Eagle's Medium) (Gibco) with 10% fetal bovine serum (FBS), 1% L-Glutamine and 1% Penicillin streptomycin (Hyclone). Cells were incubated at 37°C and humidified conditions with 5% CO_2 and routinely passaged every 4-5 days. Cells reaching 80% density were seeded into six-well plates (1×10^5). When the cells became monolayer, the control group was treated with fresh medium and the other groups were treated with HPL for 48 hrs.

MTT assay: The effects of HPL on cultured cancer cell proliferation were determined using the MTT assay. Cells were seeded into 96-well plates (1×10^4). After 48 hrs of exposure to different concentrations of HPL (5, 20, 40 and 80 µg mL^{-1}), the cell culture medium was discarded. Cells were incubated with 100 µL of MTT solution (dissolved in 0.5 mg mL^{-1} DMEM) at 37°C and 5% CO_2 for 2 hrs. Then, the MTT solution was discarded and 100 µL of DMSO was added to each well. Plates were read at 550 nm for optical density (OD) by a microplate reader (EL800, Bio-Tek Instruments, Inc.). Optical density ratio averages of wells obtained from each measurement result to the control group (HPL free) was calculated as viability.

Cell homogenization: Cells treated with HPL 48 hrs in six-well plates were taken into tubes with a volume of 15 mL. After the tubes were centrifuged at 2000 rpm at 4°C for 10 min, the liquid part was removed. After 5 mL of phosphate-buffered saline (PBS) was added to the tubes and centrifuged at 4°C at 2000 rpm for 10 min, the PBS was removed. The RIPA buffer (radio-immunoprecipitation assay) of 250 µL, PMSF (Phenylmethylsulfonyl fluoride, 200 mm) of 2.5 µL, sodium vanadate (100 mm) of 2.5 µL and protease inhibitor of 2.5 µL were added to the cells. Homogenates were obtained from cells using an ultrasonic homogenizer on ice. Homogenates were centrifuged at 10000 rpm for 10 min. Supernatants were taken and the remaining sediments (pellets) were discarded.

Enzyme-Linked Immunosorbent Assay (ELISA Assay): All ELISA kits were purchased from Abcam and performed according to the manufacturer's protocols.

Statistical analysis: Student's t-test was used to determine whether the protein expressions and MTT levels were statistically significant between the groups. The differences between the groups were considered statistically significant at the $p<0.05$ level.

RESULTS

The Bax, Bcl-2 and caspase 3 are apoptotic mediators, their intracellular transformation gives information about whether cells are undergoing apoptosis. The treatment of 20 µg mL^{-1} HPL for 48 hrs significantly increased the expression of proapoptotic protein, Bax (Fig. 1) and cleaved-caspase 3 (Fig. 2), in MCF-7 cells ($p<0.05$). The HPL treatment also significantly reduced the expression of Bcl-2 (Fig. 3) protein which is anti-apoptotic in MCF-7 cells ($p<0.05$). The current study results showed that HPL application increased the expression of apoptotic mediators and decreased antiapoptotic mediators. The HPL also decreased the viability of MCF-7 cells by dose-dependent (Fig. 4). The viability test also showed that the cells entered the apoptosis process. The HPL Treatment decreased the activity of p-ERK, p-JNK, p-AKT and p-mTOR (Fig. 5a-d) in MCF-7 cells too. The AKT/mTOR signalization pathway plays a role in cell growth and cell cycle progression. Inhibition of this pathway indirectly contributes to apoptosis. The MAPK signaling pathway consists of RAF, MEK and ERK proteins, which are essential for proliferation in many cancer cells as well as in normal cells. In normal cells,

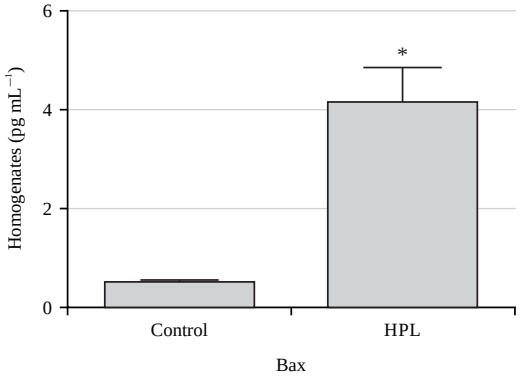

Fig. 1: HPL treatment increased bax expression and the amount of bax in the homogenates is shown as pg mL^{-1}

*$p<0.05$ when the expression levels are compared with the control

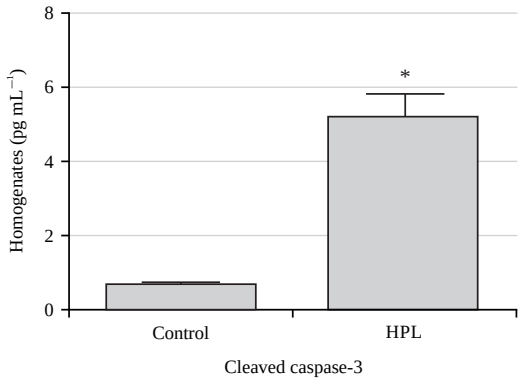

Fig. 2: HPL treatment increased activity of caspase-3 and the amount of cleaved caspase-3 in the homogenates is shown as pg mL^{-1}

*$p<0.05$ when the expression levels are compared with the control

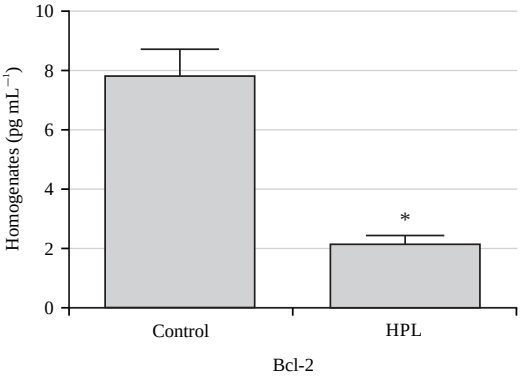

Fig. 3: HPL treatment reduced Bcl-2 expression and the amount of Bcl-2 in the homogenates is shown as pg mL^{-1}

*$p<0.05$ when the expression levels are compared with the control

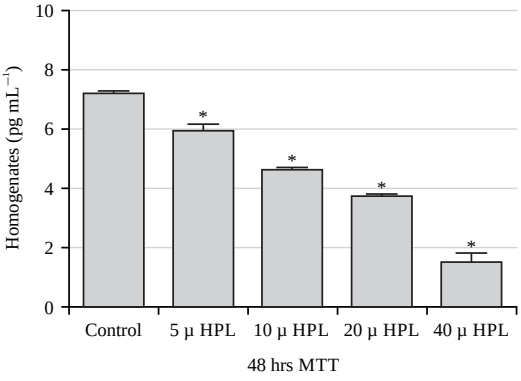

Fig. 4: HPL treatment inhibited the viability of MCF-7 cells by dose-dependent

*$p<0.05$ HPL-treated groups are compared with the control

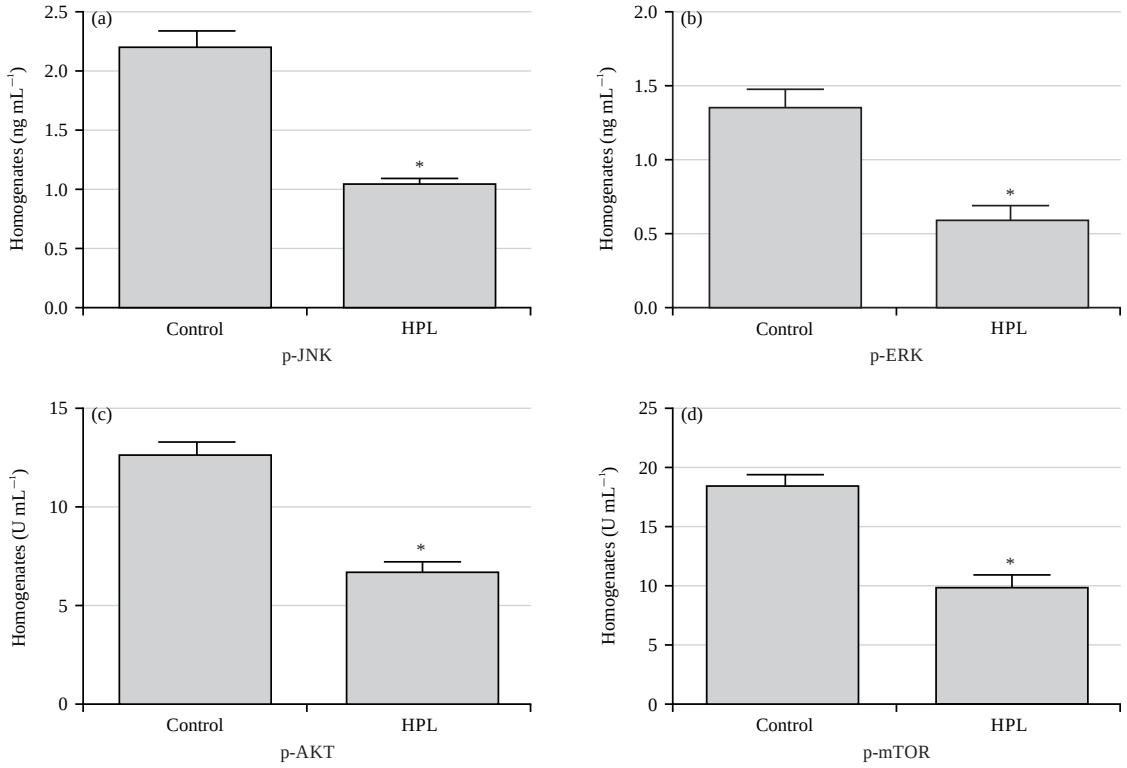

Fig. 5(a-d): HPL treatment decreased the activity of (a) p-JNK, (b) p-ERK, (c) p-AKT and (d) p-mTOR in MCF-7 cells, the amount of p-ERK and p-JNK in the homogenates is shown as ng mL^{-1} and the amount of p-AKT and p-mTOR in the homogenates is shown as U mL^{-1}

*$p<0.05$ when the expression levels are compared with the control

they lead to the activation of gene expression by transmitting signals generated by growth factor activated Receptor Tyrosine Kinases (RTKs) to the nucleus.

DISCUSSION

This study showed that HPL reduces the survival of MCF-7 cells, induces apoptotic pathway mediators and inhibits the AKT/mTOR and MAPK pathways.

The AKT/mTOR pathway, which plays an important role in the regulation of cell survival and proliferation, is thought to be effective in many types of cancer, including breast cancer[7,10]. Key genes in the pathway such as PIK3CA, AKT1, AKT2, PTEN and PI3K/AKT are mutated. These genes are activated in more than 70% of breast cancers[11]. In addition, aberrant activation of the AKT/mTOR signaling pathway is known to play a role in trastuzumab resistance in HER2-overexpressing breast cancer. It is anticipated that the combination of inhibitors of this signaling pathway with trastuzumab may increase anticancer activity in resistant cells. Therefore, it is stated that different strategies targeting the AKT/mTOR pathway are important in the treatment of breast cancer[12].

In this study, whole extracts of HPL were used and studies may be conducted to develop new drugs by revealing the molecules that content of this plant are responsible for these effects. This study also is the first to demonstrate molecularly the apoptotic effects of HPL on breast cancer. In addition, there is no study in the literature that revealed the effects of HPL on AKT/mTOR and MAPK signaling pathways. The results also showed the importance of these pathways in anticancer activity. In the methodology of the study, a few pathway mediators were analyzed. Apoptotic, MAPK and AKT/mTOR pathway mediators are very abundant. Studying all of them can fully reveal the mechanisms underlying the effects of this plant. The effect of HPL *in vitro* has been demonstrated. This study will shed light on *in vivo* studies on this plant.

Inhibiting of AKT/mTOR and MAPK pathway by HPL may reduce drug resistance and also reduce the side effects of classical chemotherapeutics. Some studies have shown that subspecies of *Haplophyllum* are protective against oxidative stress[13]. Most chemotherapeutics have oxidant activity[14-16].

Breast cancer ranks second in cancer-related deaths in women. The aim of treatment was to minimize or eliminate relapse, resistance and toxic effects while providing patients with maximum benefit from the treatment and at the same time to ensure that patients have a good quality of life. One of the biggest challenges in its treatment is the heterogeneous nature of breast cancer, which plays an important role in the selection of therapeutic approaches. Clinical response varies widely among different breast cancer subtypes due to its heterogeneous nature. Due to the existence of many subtypes and the genetic, epigenetic and translational changes that occur, there is no single standard treatment approach in the treatment of breast cancer. In addition, new treatment approaches are tried to be developed due to reasons such as resistance to existing treatment approaches and recurrence of the tumor after treatment. For this purpose, it is necessary to develop new targeted agents such as nanoparticle-based carriers that can target tumor tissue without damaging healthy tissue, inhibition of signaling pathways that mediate resistance development, treatment strategies that may respond to each different resistance mechanism and AKT/mTOR inhibitors for the treatment of specific molecular subtypes.

CONCLUSION

This study is a pioneering study in which the extract of the plant species *Haplophyllum buxbaumii* was tested. In line with the data obtained, it was determined that this plant has specific anticancer activity and AKT/mTOR and MAPK pathways inhibitory effect. When we analyze the data obtained in this study, we believe that the plant extracts of *Haplophyllum buxbaumii* will shed light on innovative and original drug studies in the treatment of breast cancer.

SIGNIFICANCE STATEMENT

This study revealed the apoptotic effect of the HPL in human breast cancer cell line MCF-7 and also showed that HPL inhibited AKT/mTOR and MAPK pathways. This study will help reveal the importance of alternative plant-based medicine based on HPL in the treatment of human breast cancer. Thus, a new theory can be reached that HPL has an apoptotic effect on human breast cancer and is a potential inhibitor of the AKT/mTOR and MAPK pathways.

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