

Investigation of Apoptotic Effects of *Hypericum perforatum* Extract on Breast Cancer Cell Line

Harun ALP^{1,a}, Hidayet TUTUN^{2,b}, Halil Mahir KAPLAN^{3,c}, Ergin ŞİNGİRİK^{4,d}, Levent ALTINTAŞ^{5,e,*}

¹Hatay Mustafa Kemal University, Tayfur Ata Sokmen Medicine Faculty, Medical Pharmacology Department, Hatay, Turkey

²Burdur Mehmet Akif Ersoy University, Veterinary Faculty, Pharmacology and Toxicology Department, Burdur, Turkey

³Cukurova University, Medicine Faculty, Pharmacology Department, Adana, Turkey

⁴Cukurova University, Medicine Faculty, Medical Pharmacology Department, Adana, Turkey

⁵Ankara University, Veterinary Faculty, Pharmacology and Toxicology Department, Ankara, Turkey

^aORCID: 0000-0002-9799-9556, ^bORCID: 0000-0001-9512-8637, ^cORCID: 0000-0003-3921-4957,

^dORCID: 0000-0003-1219-6744, ^eORCID: 0000-0002-5148-723X

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Abstract: *Hypericum perforatum* has biological active contents affecting a variety of proteins such as caspase-3, bcl-2, and bax, which mediate apoptosis known as programmed cell death and exerting anti-inflammatory effect. Apoptotic pathways are important for cancers, chemotherapeutic resistance, and cancer development. Anti-inflammatory agents are also a potential target for cancer. Therefore, it was aimed to investigate the activity of caspase-3 and the expressions of bcl-2, bax, wee 1, gadd153, grp78, AIF, iNOS, COX-2, cPLA2, and NF-κB in *H. perforatum* extract-treated breast cancer (BC) cells in this study. The activity of caspase-3 and the expressions of these proteins were determined in the cells by ELISA. The HP extract increased the activity of caspase-3 and the expressions of bax, wee 1, gadd153, grp78 and AIF, and decreased the expressions of bcl-2, COX-2, iNOS, cPLA2 and NF-κB in the BC cells. In the light of these findings, HP extract could help to inhibit growth of BC cells and its anti-inflammatory effect may contribute this effect.

Keywords: *Hypericum perforatum*, Breast cancer, Apoptosis, Inflammation.

Meme Kanseri Hücre Hattında *Hypericum perforatum* Ekstresinin Apoptotik Etkilerinin Araştırılması

Özet: Kantaron otu (*Hypericum perforatum*), programlanmış hücre ölümü olarak bilinen apoptoza aracılık eden kaspaz-3, bax ve bcl-2 gibi çeşitli proteinleri etkileyen ve antiinflamatuar etki gösteren biyolojik aktif içeriğe sahiptir. Apoptotik yollar kanserler, kemoterapötik direnç ve kanser gelişimi için önemlidir. Ayrıca, antiinflamatuar ajanlar kanser için bir potansiyel hedeftir. Bundan dolayı, bu çalışmada *H. perforatum* ekstresi ile muamele edilmiş meme kanseri hücrelerinde kaspaz-3'ün aktivitesinin ve bax, bcl-2, wee 1, gadd153, grp78, AIF, iNOS, COX-2, cPLA2 ve NF-κB ekspresyonlarının araştırılması amaçlandı. Hücrelerde kaspaz-3'ün aktivitesi ve proteinlerin ekspresyonları ELISA ile belirlendi. Ekstrakt, göğüs kanseri hücrelerinde kaspaz-3 aktivitesini ve bax, wee 1, gadd153, grp78 ve AIF ekspresyonunu arttırdı ve bcl-2, iNOS, COX-2, cPLA2 ve NF-κB ekspresyonunu azalttı. Bu bulgular ışığında, *H. perforatum* ekstresi, meme kanseri hücrelerinin büyümesini önlemeye yardımcı olabilir ve ekstrenin antiinflamatuar etkisi, bu etkiye katkıda bulunabilir.

Anahtar Kelimeler: *Hypericum perforatum*, Meme kanseri, Apoptozis, İnflamasyon.

Introduction

Breast cancer (BC) is one of the most common causes of cancer-related deaths in women after skin cancer and lung cancer. The incidence of BC in women is between 2% and 11% (Power et al., 2018; WCRF, 2018). Conventional therapy may include surgery, chemotherapy, hormonal therapy and radiotherapy for this cancer. However, patients with BC prefer complementary medicine defined as non-conventional treatments and phototherapy to stimulate their healing, due to the failure standard therapy (Sivalingam and Tharmarajah, 2018).

Hypericum perforatum (HP) is a plant that grows in the wild and has been usually used as a treatment for a variety of disease such as depression, wounds, burns, eczema (Efferth and

Koch, 2011; Butterweck, 2003). Quercetin, quercitrin, rutin, hypericin, kaempferol, biapigenin, hyperforin and hyperoside are main active components of HP (Oliveira et al., 2016). It has been demonstrated that hyperforin suppressed the activities of 5-lipoxygenase and cyclooxygenase (COX) enzymes which play a pivotal roles in regulation of inflammatory and immune responses (Albert et al., 2002). In another study on the anti-inflammatory effects of hypericin and hyperforin, it has been reported that these substrates showed anti-inflammatory activity by decreasing prostaglandin E₂ (PGE₂) and nitric oxide (NO) production and COX-2 and inducible nitric oxide synthase (iNOS) gene expression (Berköz et al.,

2018). HP has been reported to be effective antioxidant, antitumor and antimicrobial activities due to its flavonoid and some other phenolic components (Cakir et al., 2003; Milosevic et al., 2007; Pia Schiavone et al., 2014; Tungmunnithum et al., 2018). Several studies demonstrated that HP extracts and its isolated active substances such as apigenin, hyperforin, and epigallocatechin induced apoptosis through activation of caspase-3, upregulations of tumor protein p53, BH3-only protein Noxa and bax proteins and downregulation of antiapoptotic bcl-2 protein in a kind of cell lines (Gupta et al., 2004; Izol et al., 2019; Madunić et al., 2018; Merhi et al., 2011; Zaher et al., 2012). Also, there have several other proteins including nuclear factor kappa B (NF-κB), cytosolic phospholipase A2 (cPLA2), wee 1, Bid, chk2, p53, Grp78, and gadd153 having roles in regulation of cell growth and proliferation and inflammation (Baldwin, 2001; Lu et al., 2010; Yarla et al., 2015). HP extract has been reported to suppress the cell growth and induce apoptosis of human BC cells (You et al., 2018). Therefore, the aim of the present study was to investigate the effects of HP extract on apoptotic and inflammatory pathways in human BC cells.

Materials and Methods

Extraction of *Hypericum perforatum*: HP was obtained in the region of Kel Mountain in Hatay province of Turkey (June in 2017). Aerial parts and flowers of the plants were dried in incubator for 24 hours. The dried parts were powdered by using a blender and the powdered materials (40 g) were extracted with 80% ethyl alcohol solution (100 ml) as a solvent overnight on a shaker and then filtered. The alcohol of the extracts was evaporated via a rotary evaporator (IKA RV 10 basic, IKA company, Germany) to obtain crude extract. HP extract to be used in the tests was prepared with Eagle's minimal essential medium (EMEM, Gibco) at concentration of 0.25% (Ferguson et al., 2011).

Cell Culture: Human BC cells (MCF-7) were provided from ATCC. MCF-7 were cultured in the EMEM with 0.01 mg/ml human recombinant insulin and 10% FBS. Cells were cultivated at 37°C with 5% CO₂ in humidified atmosphere.

Cell Viability Assay: Cytotoxicity of the extract was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma), reduced to purple formazan in the living cells (Mosmann, 1983). The MTT was dissolved in PBS to prepare stock solution (final concentration 5 mg/ml). The MCF-7 were seeded at a concentration of 1x10⁴ cells each well of 96-well plate for overnight. Then, the cells were incubated with 0.25% HP extract for 48 h. After incubation, MTT reagent was added to

each well at final concentration of 0.5 mg/ml for 2 h at 37°C. The supernatants were carefully removed and 200 µl of DMSO (Sigma-Aldrich) was added to each well to dissolve the formazan crystals. The quantity of formazan was measured at 570 nm using a plate reading UV spectrophotometer (Rayto Life and Analytical Sciences, China).

Extract Treatment and Cell Homogenization: MCF-7 were seeded in 75 m² cell culture flasks (n=12) with 2x10⁶ cells. They were incubated at 37°C and 5% CO₂ incubator. Six flasks treated with 0.25% HP extract for 48 h and six flasks without any application (control group) were used in the study. After the MCF-7 were exposed to 0.25% HP extract for 48 h, they were washed in PBS and lysed in RIPA Lysis Buffer (89900, Thermo Scientific) on ice for 15 min. They were centrifuged at 13000 rpm for 5 min at 4°C, supernatants were collected into new microtubes, and pellets were discarded. Supernatants were used for ELISA tests.

Protein Quantification: Protein quantification was performed for standardization of ELISA assays. Bradford protein assay was used to determine the concentration of total protein in the homogenized cells. Seven dilution of Bovine serum albumin (1 µg/ml, 2 µg/ml, 3 µg/ml, 5 µg/ml, 7 µg/ml, 8 µg/ml and 10 µg/ml) standards were prepared to create standard curve used to quantify the protein concentration in the samples by comparison to the curve. 10 µl was taken from every sample and added distilled water to make 100 µl total volume. One milliliter Bradford solution was added to each standard and sample, mixed gently by vortexing and absorbance at 595 nm were measured using a spectrophotometer (Rayto Life Reader, China). Protein concentration (µg/µl) of samples was quantified according to the standard curve plotted in Prism software (GraphPad Software, USA).

ELISA Test: The activity of caspase-3 and expressions of bcl-2, bax, wee 1, gadd153, grp78, AIF, iNOS, cPLA2, COX-2, and NF-κB protein were detected by ELISA kits purchased from Shenzhen Genesis Technology (Guangdong, China) according to the manufacture's protocols.

Statistical Analyzes: For the comparison of parameters from control and the extract treated group unpaired Student's t test was used. Data were presented as means ± SEM. P values <0.05 were considered as statistically significant.

Results

We evaluated apoptotic and inflammatory pathways in this study. Treatment of cancer cells with HP extract inhibited the pathways of inflammation while activating the apoptotic pathways. HP extract increased activity of caspase-3

(Fig. 1A) and expressions of bax (Fig. 1B), wee 1, gadd153, grp78, and AIF (Table 1) which are apoptotic protein and decreased bcl-2 (Fig. 1C) which is antiapoptotic protein in MCF-7 cells. It also decreased expressions of iNOS (Fig. 1D), COX-2 (Fig. 1E), cPLA2 (Fig. 1F), and NF-κB (Fig. 1G) in the cells. In addition, the viability in the cells were evaluated by MTT test and the extract treatment for 48 h reduced viability of these cells (60±3% viability).

Table 1. Effect of HP treatment on wee 1, gadd153, grp78 and AIF expression.

Parameter (Expression)	Control	HP Treated
wee 1	0.25±0.009 pg/ml	0.95±0.017 pg/ml*
AIF	0.73±0.017 pg/ml	1.8±0.077 pg/ml*
gadd153	0.15±0.019 pg/ml	2.12±0.18 pg/ml*
grp78	0.55±0.03 pg/ml	1.35±0.25 pg/ml*

Results are presented as mean±SEM. Statistical analysis: Student t test (*: For control $P < 0.05$).

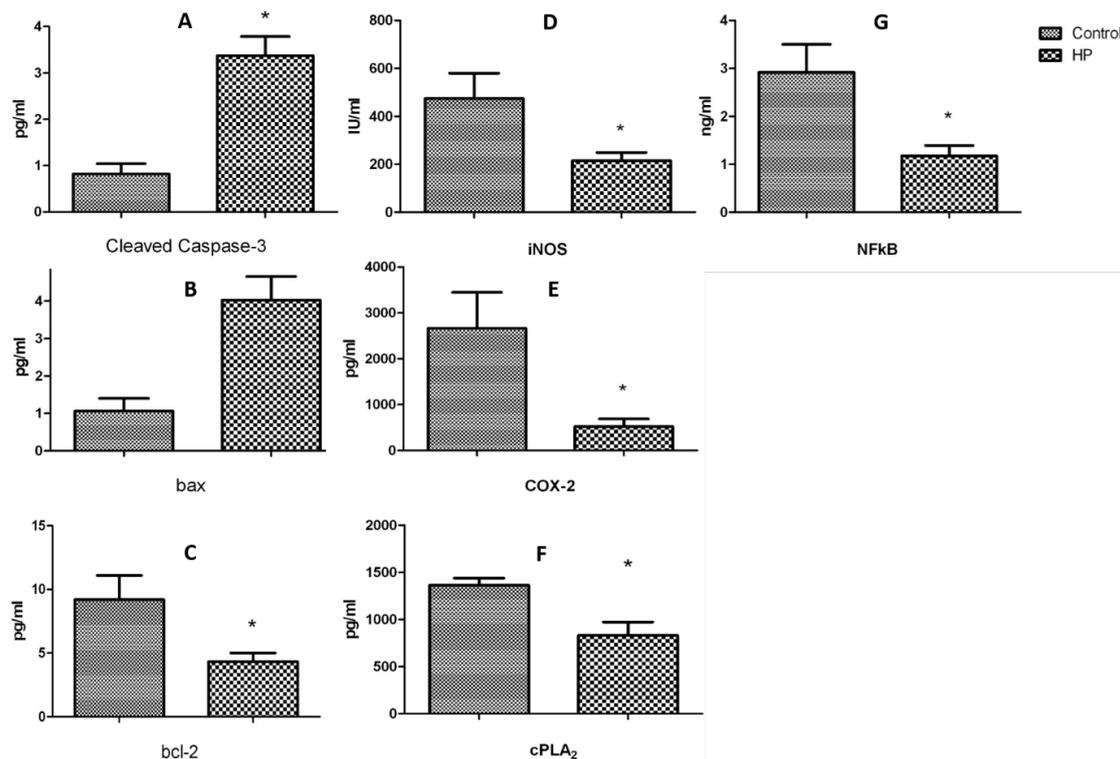


Figure 1. Effect of HP treatment on caspase-3 activity (A), bax expression (B), bcl-2 expression (C), iNOS expression (D), COX-2 expression (E), cPLA2 expression (F) and NF-κB expression (G). The data are presented as mean ± SEM. *Difference between control and HP treated group is significant with $p < 0.05$.

Discussion and Conclusions

Apoptosis known as programmed cell death is the process of elimination of undesired tissue during embryogenesis and remodeling of the tissue. Previous studies showed that some oncogenic mutations cause apoptosis, cancer initiation, progression or metastasis. It is now well documented that most cytotoxic anticancer agents induce apoptosis, and defects in apoptotic pathways contribute to treatment failure (Ma et al., 2017; Tutun et al., 2019). Inflammation has a critical role in tumor progression and many cancers occur from sites of infection, inflammation, and chronic irritation (van Kempen et al., 2006).

HP has biological active contents and has been used against some diseases for local people. The contents of HP affect caspase-3, bax and bcl-2 protein which mediate apoptosis (Gupta et al., 2004; Lu et al., 2010; Merhi et al., 2011; Zaher et al., 2012). HP extract also has anti-inflammatory effect (Albert et al., 2002). In a study, anti-inflammatory agents induced apoptosis and inhibited proliferation of human promyelocytic leukemia cell line HL-60 (Jakubikova et al., 2001). Henson and Bratton (2013) have shown that phagocytosis of apoptotic cells suppresses inflammation by inhibiting the expression of inflammatory cytokines and inducing expression of anti-inflammatory factors, including transforming growth factor (TGF)-β and PGE₂. Inhibition of COX by nonsteroidal anti-

inflammatory drugs leads to prevention of angiogenesis, inhibition of tumor cell growth and promotion of apoptosis in cancer cells (Chan, 2002; Moore and Simmons, 2000).

Hypericin, one of the active ingredients of HP, has been shown to induce apoptosis in human BC cells (Mirmalek et al., 2016). In another study, HP suppressed the growth of prostate cancer cells (Martarelli et al., 2004). Also, HP has been shown to reduce recurrence of bladder cancer (Shiverick et al., 2008). In the present study, we have revealed effects of HP extract on inflammation and apoptosis in the breast cancer cells. The findings of the present study showed that the apoptotic effects of HP on these cells could be due to its anti-inflammatory activities.

In conclusion, our study showed that HP extract activates intracellular apoptotic pathways and inhibits antiapoptotic proteins, thereby reducing inflammation of human BC. Our findings will also contribute to the development of new strategies in the treatment of cancer.

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***Corresponding Author:** Levent ALTINTAŞ

Ankara University, Veterinary Faculty, Pharmacology and Toxicology Department, Ankara.

e-mail: leventaltintas@hotmail.com.