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

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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-xB 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-xB in the BC cells. In the light of these findings, HP extract could help to inhibit grow of BC cells and its anti-inflammatory effect may contribute this effect.

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

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**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 Thamarajah, 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 (Effert 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 E\(_2\) (PGE\(_2\)) and nitric oxide (NO) production and COX-2 and inducible nitric oxide synthase (iNOS) gene expression (Berköz et al., 2019).
MCF-7 were seeded at a concentration of 1 x 10⁴ stock solution (final concentration 5 mg/ml). The MTT was dissolved in PBS to prepare 2,5-diphenyl tetrazolium bromide, Sigma), reduced 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 a 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).

**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 and expression of antiapoptotic bcl-2 protein and downregulation of proapoptotic bax, bax, wee1, gadd153, grp78, and cPLA2, while upregulations of tumor protein p53, BH3 only protein Noxa and bax proteins and downregulation of antiapoptotic blc-2 protein in a kind of cell lines (Gupta et al., 2004; Izol et al., 2019; 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), wee1, Bid, chk2, p53, Grp78, and gadd153 having roles in regulation of cell growth and proliferation and inflamation (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.

**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 cm² 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, wee1, 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 manufacturer’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).

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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