

5-Fluorouracil-induced mitochondrial oxidative cytotoxicity and apoptosis are increased in MCF-7 human breast cancer cells by TRPV1 channel activation but not *Hypericum perforatum* treatment

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Abstract 5-Fluorouracil (5-FU) is a widely used chemotherapy agent for breast cancer, although drug resistance is a critical issue regarding the use of this agent in the disease. Calcium signaling is a well-known main cause of proliferation and apoptosis in breast cancer cells. Although previous studies have implicated TRPV1 inhibitor, anticancer, and apoptotic roles of *Hypericum perforatum* (HPer) in several cells, the synergistic inhibition effects of HPer and 5-FU in cancer and the stimulation of ongoing apoptosis have not yet been clarified in MCF-7 cells. Therefore, we investigated the apoptotic and antioxidant properties of 5-FU with/without HPer through activation of TRPV1 in MCF-7 cells. The MCF-7 cells were divided into four groups: the control group, the HPer-treated group (0.3 mM), the 5-FU-treated group (25 μ M), and the 5-FU+HPer-treated group. The intracellular free calcium ion concentration ($[Ca^{2+}]_i$) increased with 5-FU treatments, but they decreased with the HPer and HPer+5-FU treatments. The $[Ca^{2+}]_i$ is further decreased in the four groups by TRPV1 channel antagonist (capsazepine and 0.01 mM) treatments. However, mitochondrial membrane

depolarization and apoptosis levels, and the PARP1, caspase 3, and caspase 9 expression levels were increased by 5-FU treatment, although the values were decreased by the HPer and 5-FU+HPer treatments. Cell viability level was also decreased by 5-FU treatment. In conclusion, antitumor and apoptosis effects of 5-FU are up-regulated by activation of TRPV1 channels, but its action was down-regulated by HPer treatment. It seems that HPer cannot be used for increasing the antitumor effect of 5-FU through modulation of the TRPV1.

Keywords Apoptosis · Breast cancer · 5-Fluorouracil · *Hypericum perforatum* · Mitochondrial oxidative stress · TRPV1 channel

Abbreviations

$[Ca^{2+}]_i$	Intracellular free Ca^{2+}
5-FU	5-Fluorouracil
CAPSN	Capsaicin
CPZP	Capsazepine
DHR 123	Dihydrorhodamine-123
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol-bis[2-aminoethyl-ether]- <i>N,N,N,N</i> -tetraacetic acid
HPer	<i>Hypericum perforatum</i>
JC-1	5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
MTT	3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide
PARP1	Poly(ADP-ribose) polymerase 1
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid 1

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Introduction

Breast cancer has the highest incidence of all cancers (28%) and the highest mortality rate worldwide for cancer types in women [1]. Chemotherapeutic agents have been used as the main therapy for treating the disease [2]. One such agent for treating breast cancer is 5-fluorouracil (5-FU) which has been widely used since the 1980s. A well-known first antitumor effect of 5-FU is blockage of DNA synthesis and cell proliferation through binding onto DNA and RNA [3]. Potentiation of apoptosis is a secondary antitumor role of 5-FU in breast cancer cells. However, clinical inadequacy of 5-FU on apoptosis and caspase production was reported due to development of resistance to 5-FU [4], and its role on molecular apoptosis mechanisms in the treatment of breast cancer cells has not been clarified yet. 5-FU is usually used in combination therapies with other antitumor agents or other compounds to increase its antitumor effect [5, 6].

Hypericum perforatum (St John's wort, HPer) is an ancient antioxidant plant. The main bioactive components of HPer are hyperforin and hypericin [7]. HPer is believed to have a number of health-promoting effects that include antioxidant, anti-carcinogenic, anti-proliferative, and apoptotic activities [8]. Extracts of HPer induced cytotoxic effect through arrest of molecular cell cycles in cultures of cell lung cancer cells [5], HT-29 colon adenocarcinoma cells [9], and of prostate cancer cells [10], and MCF-7 breast cancer cells [6]. The anti-proliferative effects of HPer extract (hyperforin) have been attributed to its direct inhibition of caspase 3 and caspase 9 activations, cell cycle arrest in different cancer cell lines [5, 9]. In addition, HPer may interfere with cancer metastasis because nuclear factor κ B (NF κ B)-regulated expression of metastatic proteins in different breast cancer cell line were shown to be down-regulated by HPer extracts such as hyperoside and hyperforin [11–13].

Several physiological functions and pathophysiological events such as mitochondrial function, breast cancer cell proliferation, and apoptosis are regulated by Ca^{2+} [14, 15]. To organize such functions, the Ca^{2+} signal must be precisely regulated. In the Ca^{2+} signal process, there are well-known channels, namely ligand- and voltage-gated calcium channels. In addition to the well-known cation channels, the transient receptor potential (TRP) channel family has been discovered within recent years. A member of the family is TRP vanilloid 1 (TRPV1) [16]. The channel is activated by different stimuli including capsaicin and oxidative stress although its activity is inhibited by specific antagonists such as capsazepine (CPZP) and 5'-iodoresiniferatoxin [17, 18]. In recent studies, we observed an inhibitor role of HPer on apoptosis, mitochondrial

oxidative stress, and the TRPV1 channel in rat neurons [19, 20] and human phagocytic cells [21]. Result of a recent study indicated that 5-FU-induced autophagic death in human hepatocarcinoma cell line (HepG2) is associated with Orail-mediated store-operated calcium entry [22]. Therefore, 5-FU may decrease cell proliferation, through increase of mitochondrial oxidative stress, apoptosis, and TRPV1 activation. Thus, it important that the relationship between changes in HPer, ROS, $[\text{Ca}^{2+}]_i$ levels, TRPV1 activation, and 5-FU-related toxicity in MCF-7 breast cancer cells becomes clarified.

Regarding this issue, especially in the case of breast cancer patients, new therapeutic strategies of 5-FU, such as combination therapy, are required. To our knowledge, there is no report on apoptosis, mitochondrial oxidative stress, and Ca^{2+} signaling through TRPV1 channels in HPer- and 5-FU-treated MCF-7 cell line. Therefore, this study was aimed to investigate if HPer could increase the anticancer effect of 5-FU through up-regulation of mitochondrial oxidative stress, apoptosis, and accumulation of intracellular $[\text{Ca}^{2+}]_i$ -induced oxidative stress in breast cancer cells.

Materials and methods

Cells and reagents

The Michigan Cancer Foundation-7 breast cancer cell line (MCF-7) was originally obtained from Şap Institute, Ministry of Food, Agriculture and Livestock, Republic of Turkey (Ankara, Turkey). Ethylene glycol-bis(2-aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 5-FU and dimethyl sulfoxide (DMSO), and Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). Fura-2/AM was purchased from Calbiochem (Darmstadt, Germany). Dihydrorhodamine-123 (DHR 123) and Tris-glycine gels were from Molecular Probes (Eugene, OR, USA). Caspase substrates [*N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (AC-LEHD-AMC) and *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (AC-LEHD-AMC)] were purchased from Bachem (Bubendorf, Switzerland). Capsaicin and CPZP were obtained from Santa Cruz Inc. (Istanbul, Turkey). A mitochondrial stain 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Santa Cruz (Dallas, TX, USA). Primary antibodies (rabbit anti-caspase 9, mouse anti-caspase 3, rabbit anti-PARP, and mouse monoclonal β -actin) were purchased from Cell Signaling Technology (İstanbul, Turkey) although secondary antibodies (Anti-mouse IgG and Anti-rabbit IgG antibodies conjugated with horseradish peroxidase) were obtained

from GE Healthcare (Istanbul, Turkey). Polyvinylidene difluoride membranes were purchased from Millipore Inc. (MA, USA). All organic solvents were also purchased from Santa Cruz Inc. (Istanbul, Turkey).

Hypericum perforatum

Hypericum perforatum extract was purchased from Xi'an Lyphar Biotech Co., Ltd. (Xian City, Shaanxi Province, China). The extract mainly contained $\geq 0.32\%$ total hypericin, 8.0% flavonoids, and 7.0% hyperforin.

Cell culture

MCF-7 cells were cultured in RPMI supplemented with 10% heat-activated fetal bovine serum, containing penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) at 37 °C in a humidified incubator at 37 °C, 5% CO₂, and 95% air. The RPMI medium was changed every other day. Preparation details of the cultures in the 96- or 24-well plates were described previously [23].

The cells were counted daily by removing a small volume from the tissue culture flask (filter cap, sterile, 250 ml, 75 cm²), diluting it with an equal volume of trypan blue (0.4%), and tallying viable cells (trypan blue excluding) with a cell counter (Casy Modell TT, Roche, Germany). Cultures were maintained as a suspension without shaking or stirring at a density of 1×10^6 cells per ml by dilution with fresh media [24].

Groups

Cells were seeded in 8–10 flasks at a density of 1×10^6 cells per flask (filter cap, sterile, 250 ml, 75 cm²). All cells were cultured at 37 °C. The cells were divided into four main groups as follows:

Control group The cells were not incubated with 5-FU, CPZP, and HPer but were kept in a flask containing the same cell culture medium and conditions for 48 h.

5-FU group Cells in the group were incubated with 5-FU (25 μM) for periods of 24 h [25].

HPer group Cells in the group were incubated with HPer (0.3 mM) for periods of 24 h [26].

5-FU+HPer group Cells in the group were incubated with 5-FU (25 μM) for periods of 24 h and then incubated with HPer (0.3 mM) for a further periods of 24 h.

For the [Ca²⁺]_i level assay, the cells were further treated with capsaicin (1 μM and 10 min incubation) for activation of the TRPV1 channel and when required, they were inhibited with the TRPV1 blocker CPZP (0.01 mM and 30 min incubation). The capsaicin, CPZP, HPer, and 5-FU

were dissolved in DMSO and the pH (7.2) was adjusted using KOH. 5-FU was prepared freshly but CPZP and capsaicin were stored at −33 °C. At the end of the incubations, the control and treated cells were used for the analyses of [Ca²⁺]_i levels using a plate reader and for Western blotting.

Intracellular free calcium ion ([Ca²⁺]_i) concentration measurement

The [Ca²⁺]_i concentration in the MCF7 cell was measured as previously described [24]. Briefly, cells were suspended in the standard extracellular buffer solution (in mM: 145 NaCl, 1 MgCl₂, 1 CaCl₂, 5 KCl, 10 HEPES, 10 D-glucose with the pH adjusted with KOH to 7.2) and 0.1% fatty acid free bovine serum albumin solution containing 2 μM fura2/AM for 45 min at 37 °C and treated with TRPV1 antagonist (CPZP and 0.01 mM) to inhibit Ca²⁺ entry before stimulation of TRPV1 (capsaicin and 1 μM). Fluorescence emission was monitored in an Eclipsys Spectrofluorometer (Varian Inc, Sydney, Australia) at 505 nm. Excitations in the assay were 340 and 380 nm. Fluorescence intensity of [Ca²⁺]_i level was calculated from the ratio of fura-2 fluorescence from excitation at 340 nm to that from excitation at 380 nm (F340/F380) and they were calibrated according to the method of Grynkiewicz et al. [27]. A total of 3 experiments were performed for measuring the [Ca²⁺]_i concentration.

The [Ca²⁺]_i level was measured using the integral of the rise in [Ca²⁺]_i for 100 s after addition of capsaicin [23]. The [Ca²⁺]_i concentration is reported as nM concentration with sampling at 1-s intervals, as previously described [28, 29].

Cell viability (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide, MTT) assay

To assess HPer protective effects on cell viability, we evaluated the mitochondrial activity of living cells by an MTT quantitative colorimetric assay. After treatments of 5-FU and HPer, the cells were washed and incubated with fresh medium containing MTT (0.5 mg/ml) at 37 °C for 90 min. Then, the supernatant was discarded and DMSO was added to dissolve the formazan crystals. The absorbance in each well was measured at 490 and 650 nm using a microplate reader (Infinite pro200; Tecan Austria GmbH, Groedig, Austria) [28, 29]. A total of 3 experiments ($n = 3$) was performed for the cell viability assay. The data are presented as the fold increase over the pretreatment level.

Assay for apoptosis, caspase 3 and 9 activities

The apoptosis analysis was spectrophotometrically performed with a commercial kit according to the instructions

provided by Biocolor Ltd. (Northern Ireland) and elsewhere [23, 24]. Briefly, the APOPercentage dye is actively transported into cells when the membrane of apoptotic cell loses its asymmetry and then the dye is staining apoptotic cells red, thus allowing detection of apoptosis by spectrophotometer.

The determinations of caspase 3 and caspase 9 activities were based on a method previously reported [28, 29] with minor modifications. Caspase 3 substrate (AC-DEVD-AMC) and caspase 9 substrate (AC-LEHD-AMC) cleavages were measured with the microplate reader (Infinite pro200) with excitation wavelength of 360 nm and emission at 460 nm. The data were calculated as fluorescence units/mg protein and presented as the fold increase over the pretreatment level.

Intracellular reactive oxygen species (ROS) measurement

DHR 123 is a non-fluorescent, non-charged dye that easily penetrates cell membrane. Once inside the cell, DHR 123 becomes fluorescent upon oxidation to yield rhodamine 123 (Rh 123), the fluorescence being proportional to ROS generation. The fluorescence intensity of Rh123 was measured in the microplate reader (Infinite Pro200). Excitation was set at 488 nm and emission at 543 nm [28]. The data are presented as fold increase over the pretreatment level.

Mitochondrial membrane potential determination

The change in mitochondrial membrane potential caused by 5-FU was assayed using a membrane-permeable dye (JC-1) [30]. The JC-1 dye accumulates in mitochondria in a potential-dependent manner. The green JC-1 signal was measured at the excitation wavelength of 485 nm and the emission wavelength of 535 nm, the red signal at the excitation wavelength of 540 nm and the emission wavelength of 590 nm. Fluorescence changes were analyzed using the microplate reader (Infinite Pro200). The data are presented as the fold increase over the pretreatment level.

Western blot analysis

The total protein in the supernatant was assessed spectrophotometrically (Shimadzu UV-1800, Kyoto, Japan) using Bradford reagent at 595 nm. Equivalent amounts of extracted proteins (50 µg) were separated by 10 or 12% SDS-polyacrylamide gel electrophoresis, and then electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked using 5% blocking buffer (5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20) for 1 h at room temperature. Blots were incubated overnight at 4 °C with the following primary antibodies:

rabbit anti-caspase 9, rabbit anti-caspase 3 (1:1000), and rabbit anti-PARP (1:1000). The membrane was washed 3 times with Tris-buffered saline containing 0.1% Tween 20 for 10 min and followed by incubation with appropriate secondary antibody for 2 h, and then washed again 3 times with Tris-buffered saline, 0.1% Tween 20. Relative levels of immunoreactivity were quantified using ECL Western HRP Substrate (Millipore Luminate Forte, USA) and visualization was achieved through X-ray film (GE Healthcare, Amersham Hyperfilm ECL, UK) [20]. Rabbit anti-β-actin (1:2000) was used as an internal control for the concentration of proteins loaded. The data are presented as relative density over the pretreatment level (experimental/control).

Statistical analyses

The SPSS statistical program (version 17.0, software, SPSS, Chicago, IL, USA) was used for analyzing the data. All results are expressed as mean ± standard deviation (SD). Statistical significances were detected using Mann-Whitney *U* test. *p* values of less than 0.05 were regarded as significant.

Results

Effects of 5-FU and HPer on intracellular $[Ca^{2+}]_i$ influx

$[Ca^{2+}]_i$ influx has been indicated to be important for apoptosis and cancer cell death [22–24], although HPer induced Ca^{2+} influx modulator role in several cells [20, 21, 31]. The line plots and columns of $[Ca^{2+}]_i$ as level and influx are presented in Fig. 1a, b, respectively. It is well known that not all cell lines contain capsaicin-sensitive TRPV1 cation channel and they need transfection procedures before studying the TRPV1 channel. However, the MCF-7 cell line has the TRPV1 cation channel without transfection [32]. In the MCF-7 cells, TRPV1 channels were activated by 5-FU but not by HPer incubations. The $[Ca^{2+}]_i$ influx was significantly ($p < 0.001$) higher in the 5-FU group than in the control. However, the $[Ca^{2+}]_i$ influx was significantly ($p < 0.001$) decreased by CPZP and HPer treatments and its influx was lower in the 5-FU+CPZP and 5-FU+HPer groups compared to the 5-FU alone group. The $[Ca^{2+}]_i$ influx was also significantly ($p < 0.001$) lower in the HPer and HPer+CPZP groups than in the 5-FU and 5-FU+HPer+CPZP groups.

Apoptosis and cell viability (MTT) results

The mean apoptosis and MTT values in the control, 5-FU, and 5-FU+HPer groups are shown in Fig. 2. The MTT

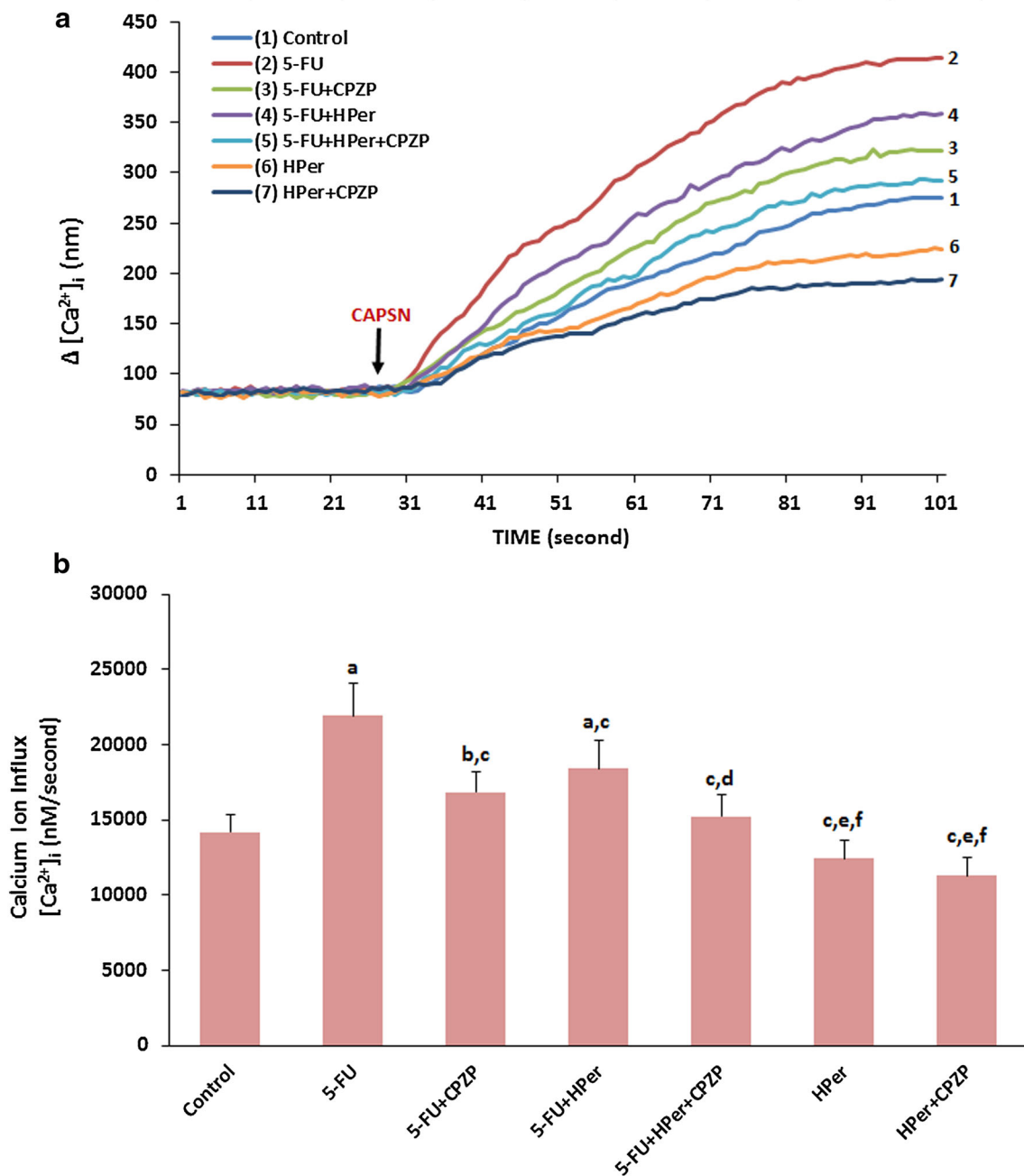


Fig. 1 In vitro effect of *Hypericum perforatum* (HPer and 0.3 mM for 24 h) and 5-FU (25 μ M for 24 h) on the free intracellular calcium ($[Ca^{2+}]_i$) levels (a) and Ca^{2+} influx (b) in MCF-7 cell ($n = 3$ and mean \pm SD). The cells are stimulated by capsaicin (CAPSN and 1 μ M for 10 min) but they were inhibited by capsazepine (CPZP and

0.01 mM for 30 min). (a) $p < 0.001$ and (b) $p < 0.05$ versus control. (c) $p < 0.001$ versus 5-FU group. (d) $p < 0.001$ and (e) $p < 0.05$ versus 5-FU+HPer group. (f) $p < 0.001$ versus 5-FU+HPer+CPZP group

level was significantly ($p < 0.001$) lower in the 5-FU groups than in the control group, although it was not increased by HPer and CPZP treatments. However, the apoptosis levels were significantly ($p < 0.001$ and 210%) higher in the 5-FU groups than in the control groups, although the levels were decreased by HPer ($\geq 240\%$) and CPZP ($\geq 250\%$) treatments ($p < 0.001$).

Caspase 3 and caspase 9 activity results

We investigated the protective effects of HPer and CPZP on the rate of programmed cell death as indicated by caspase values in the 5-FU-incubated cancer cells. The results of caspase 3 and 9 activities in the four groups are shown in Fig. 3. The caspase 3 and 9 activities in the 5-FU group

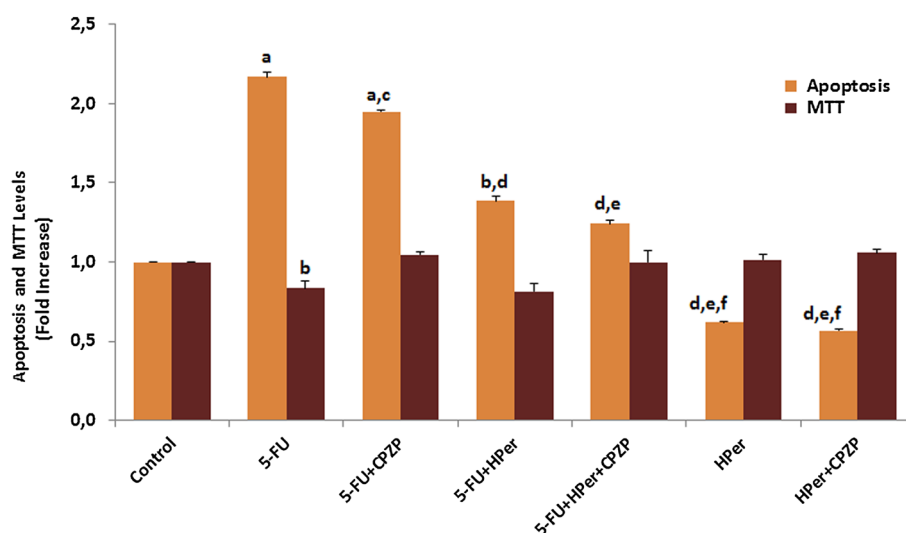


Fig. 2 Effect of *Hypericum perforatum* (HPer and 0.3 mM for 24 h) and 5-FU (25 μ M for 24 h) on apoptosis and cell viability (MTT) levels (mean \pm SD and $n = 3$). The cells were stimulated by capsaicin (CAPSN and 1 μ M for 10 min) but they were inhibited by capsazepine (CPZP and 0.01 mM for 30 min). Values are

expressed as fold increase (experimental/control). (a) $p < 0.001$ and (b) $p < 0.05$ versus control. (c) $p < 0.001$ and (d) $p < 0.05$ versus 5-FU group. (e) $p < 0.001$ versus 5-FU+CPZP group. (f) $p < 0.001$ versus 5-FU+HPer+CPZP group

were markedly ($p < 0.001$) higher than in the control and HPer groups. However, the caspase 3 and 9 activities were markedly ($p < 0.001$) lower in the 5-FU-HPer and HPer groups than in the 5-FU group only. There were effects on caspase activity of HPer treatments in the cancer cells. There was no statistical change in the caspase activities in CPZP-treated groups. On the other hand, the caspase 3 and 9 activities did not differ between 5-FU and 5-FU+CPZP groups or also between 5-FU+HPer and 5-FU+HPer+CPZP.

Intracellular ROS production and mitochondrial depolarization (JC-1) levels

In order to determine whether the potentiating effects of HPer and the 5-FU is due to a production of intracellular ROS, fluorescence (JC-1) of oxidation-sensitive intracellular probes (DHR123)-loaded MCF-7 cells were treated with HPer. The mean JC-1 and ROS levels in the four groups are shown in Fig. 4 and it can be seen that the levels of JC-1 and ROS were markedly higher ($p < 0.001$) in the

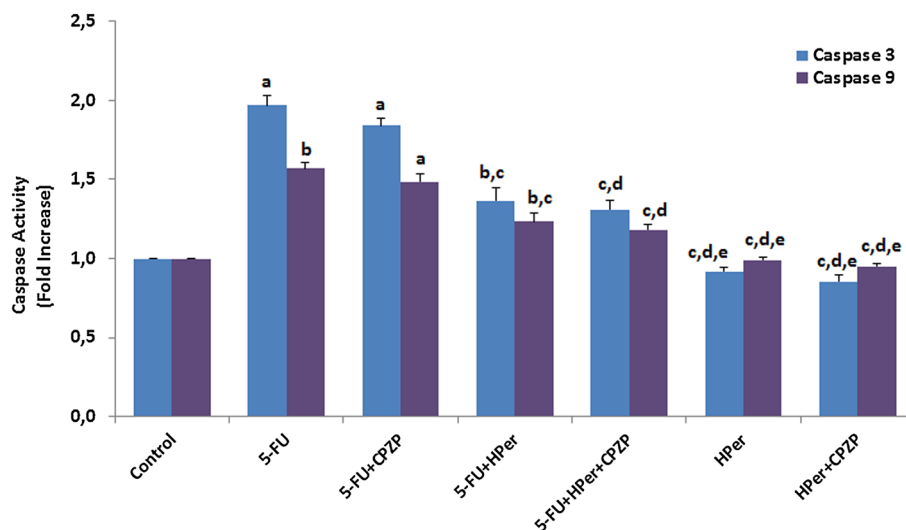


Fig. 3 Effect of *Hypericum perforatum* (HPer and 0.3 mM for 24 h) and 5-FU (25 μ M for 24 h) treatments on caspase 3 and 9 activities in the MCF-7 cells (mean \pm SD and $n = 3$). The cells were stimulated by capsaicin (CAPSN and 1 μ M for 10 min) but they were inhibited

by capsazepine (CPZP and 0.01 mM for 30 min). Values are expressed as fold increase. (a) $p < 0.001$ and (b) $p < 0.05$ versus control. (c) $p < 0.001$ versus 5-FU group. (d) $p < 0.001$ versus 5-FU+CPZP group. (e) $p < 0.001$ versus 5-FU+HPer+CPZP group

5-FU groups than those in the HPer and control groups. However, the JC-1 and ROS levels were markedly decreased ($p < 0.001$) by HPer treatments. Pretreatment with CPZP did not add to the effect on the JC-1 and ROS levels found in the 5-FU+HPer groups and their levels did not differ in the 5-FU and 5-FU+CPZP or between the 5-FU+HPer and 5-FU+HPer+CPZP groups.

Caspase 3, caspase 9, and poly(ADP-ribose) polymerase (PARP) expression levels

Upon apoptotic stimulation, cytochrome c released from mitochondria becomes associated with procaspases 3 and 9/Apaf 1 and then the procaspases are converted to active caspases 3 and 9. In the current study, we assayed active caspase 3 and 9 expression levels as an indicator of apoptosis (Fig. 5). The caspase 3 and 9 expression levels were markedly higher ($p < 0.05$) in the 5-FU groups than those in the control group. The caspase 3 and 9 expression levels were decreased in the HPer ($p < 0.001$) and 5-FU+HPer ($p < 0.05$) groups compared with the 5-FU alone group.

PARP1 is an abundant enzyme present in cells that indicates and signals damage to DNA repair mechanisms. We measured PARP1 expression level as an indicator of damage to DNA repair mechanisms in the four groups (Fig. 6). The PARP1 expression level was significantly ($p < 0.05$) higher in 5-FU groups than that in the control group. The PARP1 expression level was remarkably decreased in the HPer ($p < 0.01$) and 5-FU+HPer ($p < 0.05$) groups compared with the 5-FU group.

Discussion

Resistance to chemotherapy can develop during treatment of breast cancers. Therefore, development of an adjuvant therapy with 5-FU in the treatment of breast cancer has great interest. HPer is a traditional medicine that has been used from ancient times in treatments of human neurological disease and now is known to be rich in antioxidants [8]. It has been used for treatment of cancer in a combination with chemotherapeutic agents but not with 5-FU [6, 9, 10]. HPer was tested on apoptosis and cell viability values in cisplatin-exposed MCF-7 cells [6, 33], but its molecular mechanisms of action in the treatment of breast cancer cells have not been clarified yet. The results of the current study clearly indicate that anti-apoptosis and oxidant effects of 5-FU involved activation of TRPV1 channel, although these effects were reduced by HPer treatment through inhibition of the TRPV1.

Oxidative stress induces an irreversible effect on cellular components such as nucleic acids, lipids, and proteins [15] in which the intracellular level of ROS is increased. Chemotherapeutic agents, such as 5-FU, kill cancer cells through over production of intracellular ROS [34]. Overload of Ca^{2+} entry induces excessive mitochondrial ROS production, mitochondrial membrane depolarization, and caspase 3 and 9 activations [28, 30]. TRPV1 channels are activated by different stimuli including capsaicin and oxidative stress in different cell types including MCF-7 [35]. Therefore, mitochondria are a target for cancer prevention and chemotherapy because chemotherapeutic agents may modulate or interfere with mitochondrial

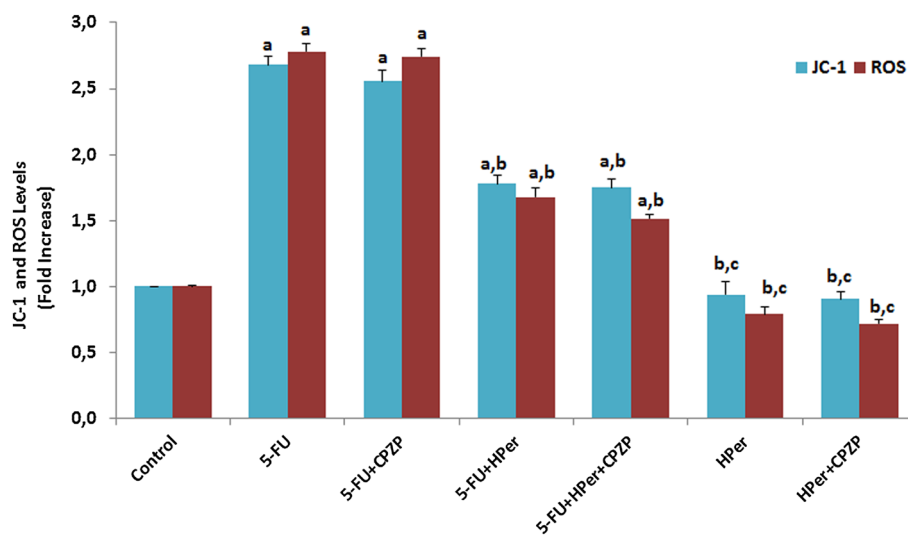


Fig. 4 Effect of *Hypericum perforatum* (HPer and 0.3 mM for 24 h) and 5-FU (25 μ M for 24 h) treatments on mitochondrial membrane depolarization (JC-1) and intracellular ROS production in the MCF-7 cells (mean \pm SD and $n = 3$). The cells are stimulated by capsaicin (CAPSN and 1 μ M for 10 min) but they were inhibited by

capsazepine (CPZP and 0.01 mM for 30 min). Values were expressed as fold increase. (a) $p < 0.001$ versus control. (b) $p < 0.001$ versus 5-FU and 5-FU+CPZP groups. (c) $p < 0.001$ versus 5-FU+HPer and 5-FU+HPer+CPZP groups

Fig. 5 Effect of *Hypericum perforatum* (HPer and 0.3 mM for 24 h) and 5-FU (25 μ M for 24 h) treatments on caspase 3 and 9 expression levels in MCF-7 cells (mean \pm SD and $n = 3$). Values were expressed as relative density over the pretreatment level. (a) $p < 0.05$ versus groups control. (b) $p < 0.001$ versus 5-FU and 5-FU+HPer groups

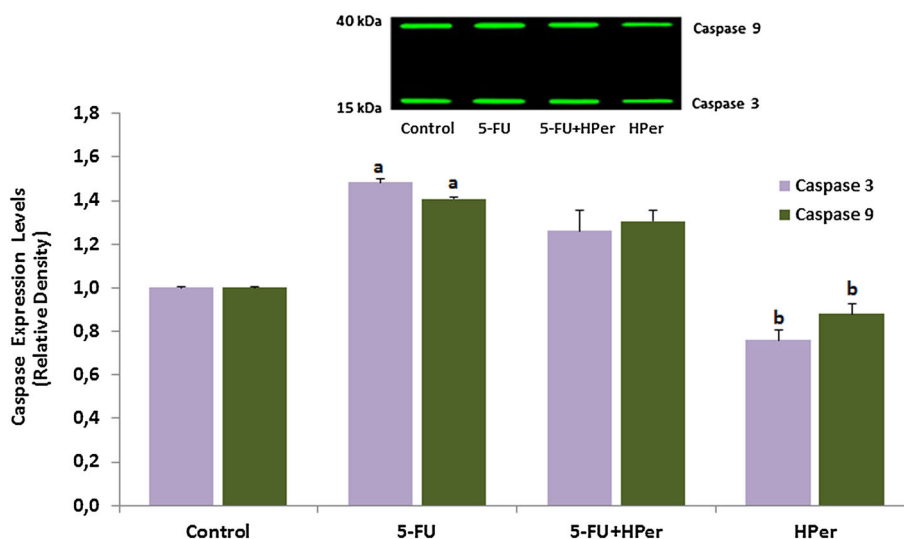
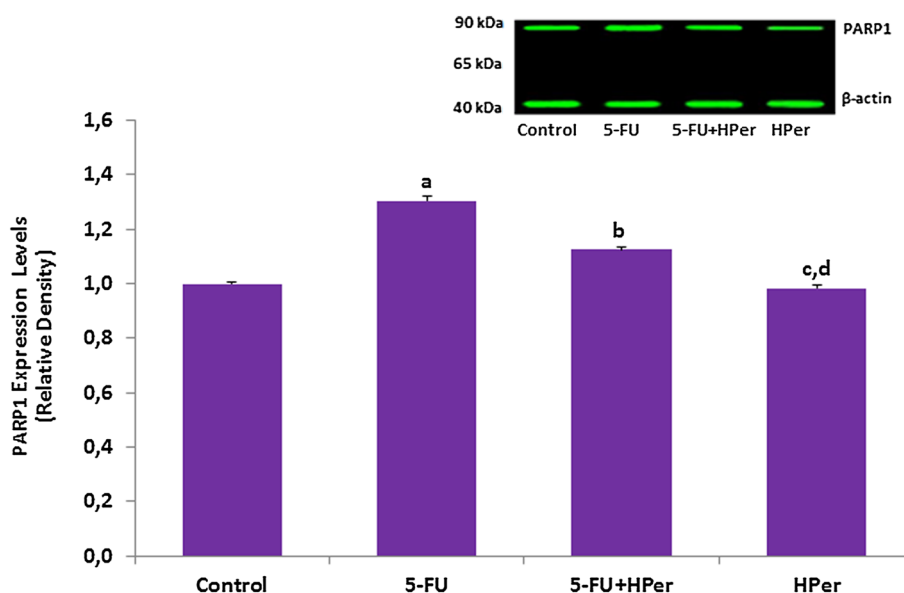


Fig. 6 Effect of *Hypericum perforatum* (HPer and 0.3 mM for 24 h) and 5-FU (25 μ M for 24 h) treatments on PARP1 expression level in MCF-7 cells (mean \pm SD and $n = 3$). Values were expressed as relative density over the pretreatment level. (a) $p < 0.05$ versus groups control. (b) $p < 0.05$ and (d) $p < 0.01$ versus 5-FU group. (c) $p < 0.05$ versus 5-FU+HPer group



functions to promote mitochondrial membrane permeability and cell death [4, 28, 36]. Chemotherapeutic agent-induced excessive Ca^{2+} entry through activation of TRPV1 changes membrane structure and functions, promotes excessive intracellular ROS production and depolarization of the mitochondria of intact cells, and induces apoptosis in the MCF-7 cells [23, 24]. In the current study, 5-FU induced excessive Ca^{2+} entry through activation of TRPV1 and raised intracellular ROS production and depolarization of the mitochondria of intact cells, and induced apoptosis and caspase 3 and 9 activations in the MCF-7 cells. The inhibitor role of HPer on the TRPV1 channel, apoptosis, mitochondrial membrane depolarization, and intracellular ROS production in rat neurons and human neutrophils has been reported [19–21, 37]. Similar molecular pathways have now been observed in the current study and we have

shown the inhibitory role of HPer in the 5-FU-induced apoptosis, mitochondrial membrane depolarization, intracellular ROS production, caspase 3 and 9 expression levels in MCF-7 cells through inhibition of TRPV1 channel.

The results of the present study show that 5-FU-induced apoptosis and caspase 3 and 9 expression levels were decreased in the MCF-7 cell line by HPer treatment although MTT levels were increased by the treatment. Studies of HPer effects on apoptosis in cancer cell lines are somewhat limited and have produced conflicting results. Similarly, Mirmalek et al. [6] reported that the apoptosis levels indicated by annexin V in MCF-7 cells was lower in a hypericin-treated group (5 μ g/ml and 24 h incubation) than in a cisplatin group. The result of another study indicated that a low concentration (100 nM) of hypericin did not inhibit an apoptotic effect in the human fibroblasts

cell line, although treatment with normal concentration (10 μM) of hypericin failed to induce apoptosis [38]. However, the results reported by Roscetti et al. [33] indicated that purified hypericin (used in 24 and 48 h incubations) has no inhibitory effect on cell viability and apoptosis in a human erythroleukemic cell line, although a methanolic extract of HPer induced apoptotic effect in the cells. Contrary to our findings, Acar et al. [11] investigated the apoptotic effect of two different doses (1.5 and 7.5 $\mu\text{g}/\text{ml}$) of hypericin in MCF-7 cells and found an apoptotic action of 7.5 $\mu\text{g}/\text{ml}$ of hypericin but not by 1.5 $\mu\text{g}/\text{ml}$. Özen et al. [12] investigated the dose-dependent effect of hypericin on apoptosis in blood cancer (HL-60) cells and were unable to demonstrate any apoptotic effect up to a dose of 5 μM dose. It seems that an apoptotic effect of HPer in cancer cells occurs in a dose-dependent manner but can be cell specific.

The critical role of $[\text{Ca}^{2+}]_i$ levels in regulating cancer cell proliferation has been well documented in breast cancer cells [39]. Activation of the TRPV1 channel induces Ca^{2+} entry from the extracellular fluid and evokes a transient increase of $[\text{Ca}^{2+}]_i$ level leading to the stimulation of intracellular ROS production and DNA damage through PARP1 activation [15, 16]. In turn, the increased ROS induces further activation of the TRPV1 channel and increase of $[\text{Ca}^{2+}]_i$ levels. In mice neurons [40, 41] and pancreatic cancer cells [42], activation of TRPV1 increases Ca^{2+} entry and apoptosis formation, whereas decrease of $[\text{Ca}^{2+}]_i$ concentration through TRPV1 treatment results in inhibition of apoptosis [16, 20, 35]. In the present study, we have provided convincing evidence that TRPV1-mediated Ca^{2+} entry stimulated 5-FU-induced apoptotic cell death in MCF-7 cells via up-regulation of mitochondrial oxidative stress, caspase, and PARP1 by a Ca^{2+} signaling molecular pathway. The apoptosis level was decreased by HPer through down-regulation of the molecular pathways. Similarly, Eriksson and Eriksson [13] reported possible interactions between hypericin and the Ca^{2+} SERCA pump.

In conclusion, 5-FU induced anti-apoptotic cell death in MCF7 breast cancer cells through stimulating Ca^{2+} entry via activation of TRPV1 channels, although HPer induced anti-apoptotic effects through inhibition of the TRPV1 channel. Therefore, stimulation of TRPV1 channel activation increased apoptotic effect of 5-FU in MCF-7 cells. However, blockade of TRPV1-mediated Ca^{2+} entry through HPer treatment may not be a potential strategy to sensitize breast cancer cells to 5-FU treatment.

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Compliance with ethical standards

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