Hypericium perforatum extract (St. John's Wort) and hypericin induce apoptosis in leukemic HL-60 cells by effecting h-TERT activity

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ABSTRACT

Hypericin is the main active component of *Hypericium perforatum* (St. John's Wort). Hypericin has been proven to have antitumoral effect in in vitro condition against solid tumors by deteriorating the mitochondrial functions. It has also anti-leukemic effect in in vitro conditions. However, there has not been any comparative study with hypericin and extract obtained from *Hypericium perforatum L*. In this study, it has been aimed to investigate the potential cytotoxic role of the extract obtained from *Hypericium perforatum* grown in Ege region on leukemic cell line, to compare the cytotoxic effects of both extract and hypericin in HL-60 cells, and to clarify the underlying mechanism(s) of this cytotoxicity. *Hypericium perforatum* extract was used in dilutions as 1/1000, 1/5000, 1/10.000, 1/50.000 and the IC_{50} value was found to be as 1/10.000 dilution. Hypericin was found to have cytotoxicity in HL-60 cells in time and dose dependent manner between the doses of 1nM to 100 μM with IC_{50} dose of 0.5 μM. Hypericin with the dose of 0.5 μM had similar cytotoxicity pattern with the cytotoxicity curve obtained with 1/10000 diluted extract. Apoptosis as an underlying mechanism of this cytoxocity was shown in HL-60 cells after incubation with IC_{50} dose of hypericin which was more remarkable at 48th hours by using acridine orange/ethidium bromide dye method. Total RNA was isolated concomittantly and h-TERT mRNA expression was analyzed at Light Cycler Real-time online polymerase chain reaction and it was found that the mRNA expression was meaningfully decreased at 48th hour of incubation of cells with hypericin. According to results of this study, we have shown that hypericin, as main cytotoxic compound of *Hypericium perforatum L*, induces apoptosis in HL-60 cells via effecting h-TERT mRNA expression

Key Words: *Hypericium perforatum* L., hypericin, acute leukemia, h-TERT

ÖZET

Sarı kantaron ekstraktı ve hiperisin HL-60 akut miyeloblastik lösemi hücre dizisinde h-TERT üzerinden apoptotik etki göstermektedir

Hiperisin, sarı kantaron (Hypericum perforatum L.) olarak bilinen bitkinin aktif bileşenlerinden birisidir. Hiperisinin antitümöral özellikleri olduğu solid organ tümörlerinin hücre dizileri ve hayvan modellerinde gösterilmiş ve bunun mitokondrial fonksiyonlarda bozulma neticesinde ortaya çıktığı gösterilmiştir. In vitro antilösemik etkisi de vardır. Ancak etki mekanizması tam olarak aydınlatılabilmiş değildir. Hiperium ekstraktı ile hiperisine ait karşılaştırmalı sitotoksisite çalışması yoktur ve sitotoksisitenin altında yatan mekanizma aydınlatılamamıştır. Bu çalışmada Ege Bölgesinde yetişen sarı kantaron otundan Hypericum perforatum ekstraktının elde edilip, bu ekstraktın HL-60 lösemik hücre dizisinde doz ve zamana bağımlı sitotoksisitesi olup olmadığını ve varsa bu sitotoksisik etkiyi hiperisinin sitotoksik özellikleri ile karşılaştırmak ve bu etkinin altında yatan mekanizmanın açıklanması amaçlanmıştır. Sarı kantaron otu ekstraktının 1/1000, 1/5000, 1/10.000, 1/50.000 dilüsyonları ile yapılan deneylerde, IC_{50} dozunun 1/10.000 dilüsyon olduğu görüldü. Hiperisin ile, 1nM ve 100 μM aralığında logaritmik artış gösteren dozlarda yapılan deneylerde de doz ve zamana bağımlı sitotoksisite olduğu ve 0.5 μM konsantrasyonunun, ekstraktın 1/10.000 dilüsyonunun gösterdiği eğriye paralel bir sitotoksisite gösterdiği tespit edildi. Hiperisinin sitotoksisite mekanizmasına yönelik olarak yapılan Acridine oranj/ethidium bromide boyamasında, 48. saatte belirgin olmak üzere IC_{50} dozunda, kontrollere oranla belirgin apoptozis varlığı saptanmıştır. Eş zamanlı olarak total RNA izole edilerek Light Cycler Real-time online polimeraz zincir reaksiyonu cihazında kit manueline uygun olarak h-TERT mRNA ekspresyonu çalışılmış ve 48. saatte belirgin ekspresyon azalması olduğu bulunmuştur. Sonuç olarak, kantaronun esas etken maddesi olan hiperisinin, HL-60 hücre dizisinde h-TERT ekspresyonunu etkileyerek apoptozisi uyardığı ortaya konmuştur.

Anahtar Sözcükler: Hypericium perforatum L., hiperisin, akut lösemi, h-TERT

INTRODUCTION

Hypericum perforatum (St. John's Wort) has traditionally been used in folk medicine, and various forms of cancers have been treated with therapies including Hypericum extracts or one of its compounds, hypericin [1]. Hypericin is a photoactive pigment synthesized by members of the plant genus Hypericum. Hypericin exhibits, in vitro, antineoplastic, antiproliferative and apoptotic activity. In many malignant cell lines, it has been proven that hypericin can induce cell death via apoptosis. Several mechanisms have been described and published as the underlying mechanisms of hypericin-induced apoptosis [1-3]. Hypericin-induced apoptosis was first described in cultured human malignant glioma cells [4]. Firstly, hypericin-mediated protein kinase C inhibition was proposed as the triggering event in this type of cell death. However, later studies in human malignant cell lines revealed that protein kinase C inhibition might not be sufficient to cause apoptosis alone, but could be only one of the possible mechanisms [1-4]. Recent studies have shown that hypericin-induced apoptosis is also associated with mitochondrial membrane depolarization in glioma cells, HeLa cells and Jurkat cells, release of cytochrome C from mitochondria of HeLa cells and activation of caspase-3 in HeLa and Jurkat cells, documenting the strong relationship between hypericin and mitochondria-involved apoptosis [4-7]. Although many studies have indicated the mitochondria-involved apoptosis could be the underlying mechanism of hypericin-induced cell death, the exact mechanism(s) remain(s) to be clarified. Furthermore, no study has been found in the literature comparing the anti-tumoral effects of *H. perforatum* L. extract and hypericin.

Telomerase is an enzyme that synthesizes and adds repetitive telomeric sequences of TTAGGG to the ends of chromosomes. The function of telomerase is to prevent the shortening of chromosomes. A human telomerase is composed of three major subunits: a hu-

man telomerase RNA subunit (hTR), a human telomerase reverse transcriptase (h-TERT), and telomerase-associated protein (TEP1). h-TERT is known to be a catalytic subunit of telomerase. h-TERT mRNA expression temporally parallels changes in telomerase activity during cellular differentiation [8]. Telomerase activity is not detectable in somatic cells except for fetal and adult testes, fetal ovary, and proliferative cells from renewal tissues, hematopoietic stem cells, lymphocytes, hair follicles and cryptic cells in the intestine. However, high telomerase activity had been detected in 85% of human malignancies (e.g. hematological malignancies). Thus, it has been thought that telomerase activity is an important marker in the carcinogenesis [8-10]. In this study, we aimed to investigate the cytotoxicity of both *H. perforatum* (St. John's Wort) extract and hypericin and the potential role of h-TERT in hypericin-induced cytotoxicity in HL-60 leukemic cells. To date, there has been no comparable published study about the cytotoxic effects of *H. perforatum* extract and hypericin in the same conditions.

MATERIALS and METHODS
Cell line

The human myelogenous leukemia cell line, HL-60, was kindly provided by Ali Ugur Ural, Gulhane Military School of Medicine-Cancer Research Center, Ankara, Turkey. The cell line was maintained in RPMI-1640 medium (Sigma Chemical Co., St. Louis, Missouri) supplemented with 1% nonessential amino acids (Sigma Chemical Co., St. Louis, Missouri), 1% L-glutamine (Sigma Chemical Co., St. Louis, Missouri), 10.000 units/mL penicillin, 10 mg/mL streptomycin (Sigma Chemical Co., St. Louis, Missouri), and 10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, Missouri). The cell line was grown in a standard cell culture incubator at 37°C, 100% relative humidity and in 5% CO_2 atmosphere.

Chemicals and reagents

H. perforatum extract was synthesized by Yasar Hisil from the Food Engineering Department-Engineering Faculty, Ege University, Izmir, Turkey, by using 1,1,1,2- tetrafluoroetha-

ne method, which was described in a detailed review published recently [11]. Analyses of *H. perforatum* extract were performed by using [gas chromatograph (GC), mass spectrometer (MS)] total ion chromatography. Hypericin, dimethylsulfoxide (DMSO), trypan blue, acridine orange, and ethidium bromide were purchased from Sigma Chemical Co., St. Louis, Missouri. Cell proliferation kits II (XTT) and h-TERT quantification kit were purchased from Roche Diagnostics GmBH, Germany.

H. perforatum extract and hypericin treatments

Cells were seeded 2-4 x 10^5 cells/mL in 24 well-plates. *H. perforatum* extract diluted in RPMI 1640 medium was used in treatments of 1/1000, 1/5000 and 1/10.000 concentrations. Hypericin was firstly dissolved in DMSO and then diluted in RPMI 1640 medium. Hypericin was used in concentrations of 1nM, 10 nM, 100 nM, 500 nM, 1 μM, 10 μM, respectively.

Cytotoxicity and cell proliferation experiments

Cytotoxicity assays and determination of IC_{50} dose of *H. perforatum* extract and hypericin in HL-60 cell line were performed by using trypan blue dye exclusion and XTT assays. XTT assay is based on the cleavage of the tetrazolium salt, which turns to orange-colored soluble formazan crystals by metabolic active cells. The absorbance values (A) proportional to the degree of cell viability determined at 492 nm were read using an automatic multiwell spectrophotometer. The negative control well was used for zeroing absorbance. The percentage of cytotoxicity was calculated using the background-corrected absorbance as follows:

$$\text{Cytotoxicity (\%):} = \frac{1- A \text{ of experiment well}}{A \text{ of positive control well}} \times 100$$

Morphologic evidence of apoptotic cells by acridine orange/ethidium bromide staining

Apoptosis was examined morphologically after staining with acridine orange and ethidium bromide by fluorescence microscopy. Cells were adjusted to the cell density to 1-5 x 10^6

cells/mL in PBS. Acridine orange and ethidium bromide (1:1) (v/v) were added to the cell suspension in final concentrations of 100 µg/mL and then cells were incubated for 30 minutes. The cellular morphology was evaluated by fluorescent microscopy. Apoptotic cells were essentially characterized by nuclear condensation of chromatin and/or nuclear fragmentation. Four hundred fifty cells were evaluated for apoptosis and/or necrosis for each sample.

Measurement of h-TERT

A quantitative measurement of h-TERT mRNA was performed with the commercially available LightCycler Telo TAGGG h-TERT Quantification Kit by using the LightCycler instrument for real-time polymerase chain reaction (RT-PCR). All subsequent quantification steps were carried out according to the manufacturer's instructions.

Statistical analysis

All analyses were set up in triplicate and the results were expressed as the mean plus or minus standard deviation.

RESULTS

The effect of H. perforatum extract on the cell viability

Treatment of HL-60 cells with *H. perforatum* extract showed a time- and dose-dependent inhibition of cell proliferation. The cell numbers (using trypan blue exclusion) as well as metabolic rates (using XTT assay) were determined. After 72 hours, the IC_{50} dose of extract was found to be 1/10.000 dilution revealed by trypan blue dye exclusion test (Figure 1A). The metabolic rates as determined by XTT assay (Figure 1B) were similar to findings obtained by trypan blue dye exclusion test.

The effect of hypericin on the cell viability

Treatment of HL-60 cells with hypericin in concentrations of 1nM, 10 nM, 100 nM, 500 nM, 1 µM, and 10 µM, respectively, showed a dose- and time-dependent inhibition of cell proliferation. By using trypan blue dye exclusion test and XTT assay, we determined the
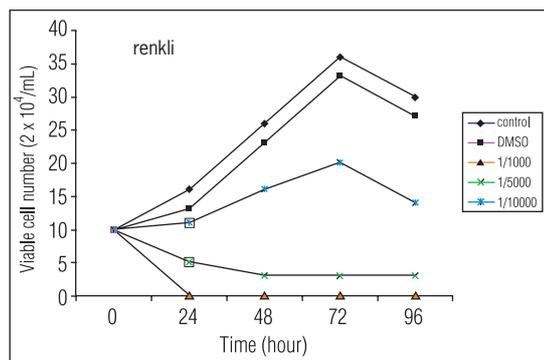

Figure 1A. The cytotoxic effect of Hypericium perforatum extract on HL-60 cells. Data were obtained by using trypan blue dye exclusion test.
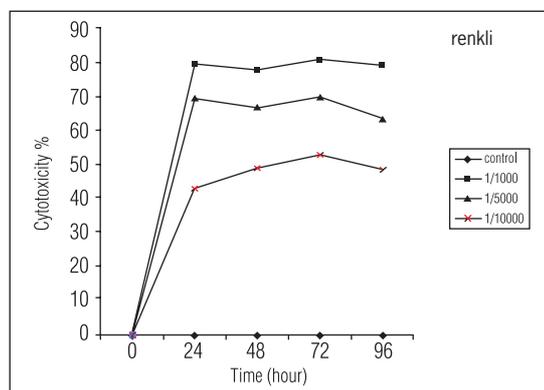

Figure 1B. The cytotoxic effect of Hypericium perforatum extract on HL-60 cells. Data were obtained by using XTT assay.

cell numbers and the metabolic rates of HL-60 cells after application of hypericin (Figures 2A, 2B). After 72 hours, the IC_{50} dose of hypericin was found to be 500 nM, and this dose value showed a parallel cytotoxicity with the 1/10.000 dilution of *H. perforatum* extract.

Identification of apoptosis with acridine orange and ethidium bromide staining

To assess the apoptotic effects, HL-60 cells were treated with hypericin using 500 nM (IC_{50} dose) and 1 µM concentrations. After application of acridine orange and ethidium bromide, fluorescence microscopy revealed nuclear changes including chromatin condensation or nuclear fragmentation. Beginning from 48 hours, especially in the 72nd hour, IC_{50} dose (500 nM) of hypericin exhibited apoptosis comparable with the controls (Figures 3A, 3B).
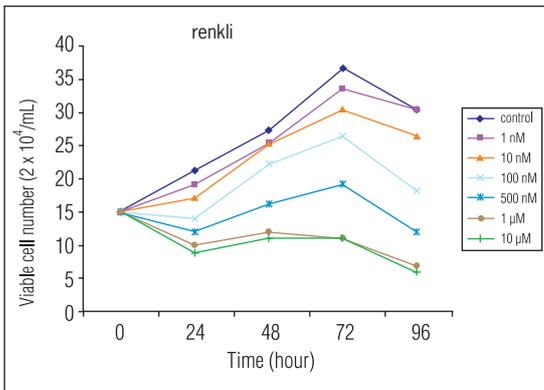
Figure 2A. The cytotoxic effect of Hypericin on HL-60 cells. Data were obtained by using trypan blue dye exclusion test.
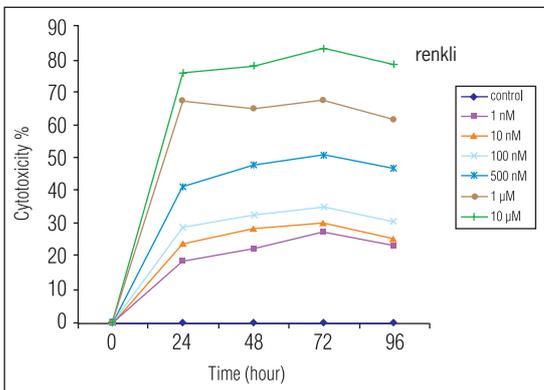

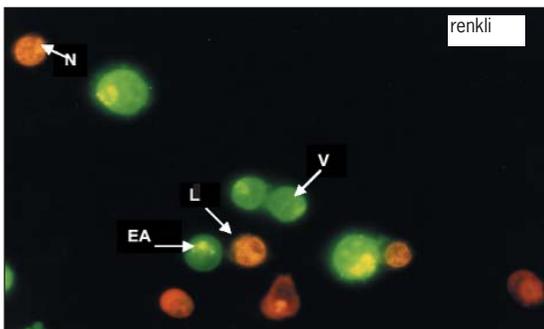**Figure 2B.** The cytotoxic effect of Hypericin on HL-60 cells. Data were obtained by using XTT assay.

Figure 3A. Fluorescence microscopy of hypericin-treated cultured HL-60 cells. Cultured cells were incubated with 0.5 μM hypericin for 48 hours. Cells were double stained with the membrane permeable fluorochrome acridine orange (green fluorescence) and DNS-binding fluorochrome ethidium bromide, which is impermeable to the normal membrane but stains the nuclei of necrotic and late apoptotic cells (red fluorescence). Note that chromatin condensation and margination as well as ruffling of nuclear membrane had already occurred in cells that did not take up ethidium bromide. (V: Viable cells, L: Late apoptotic cells, EA: Early apoptotic cells, N: Necrotic cells).
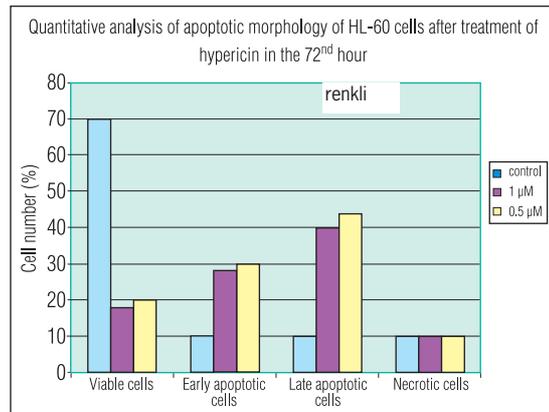

Figure 3B. Quantitative analysis of apoptotic morphology of HL-60 cells after treatment with hypericin. Minimum 500 to maximum 1000 cells were counted under the microscope to perform this analysis.

h-TERT activity assay

To investigate the mechanisms involved in hypericin-induced apoptosis in the HL-60 cell line, expression of telomeric subunit h-TERT was examined at the mRNA level using RT-PCR. It began to decrease after treatment with 500 nM dose of hypericin for 48 hours (Figure 4). The higher concentration of hypericin (1 μM) induced more remarkable decrease in the h-TERT mRNA expression, but this concentration led cells to express necrotic cell death pattern in apoptosis assay (data not shown).
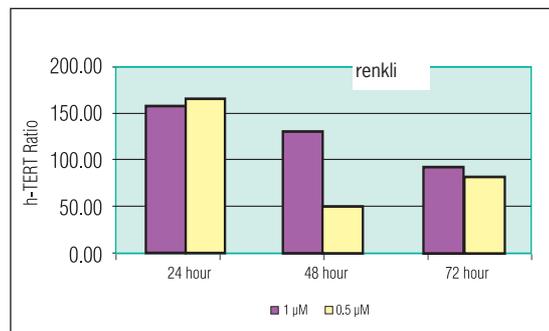

Figure 4. The quantification of h-TERT mRNA expression by using real time PCR in cells incubated with 0.5 and 1 μM hypericin for 72 hours with 24-hour intervals. Treatment of cells with 1 μM hypericin caused more prominent decrease in mRNA expression than the dose of 0.5 μM hypericin, since 1 μM induced remarkable cell death in cytotoxicity assay; apoptosis studies were performed using 0.5 μM dose of hypericin as IC_{50} value.

DISCUSSION

One of the aims of this study was to examine the cytotoxic effects of *H. perforatum* extract and hypericin. We studied the effect of an *H. perforatum* extract on the growth of leukemic HL-60 cells, and are the first to compare it with hypericin. There is no comparable study establishing the cytotoxic effects of both *H. perforatum* extract and hypericin. We found that both *H. perforatum* extract and hypericin inhibited the cell proliferation in HL-60 leukemic cell line in a time-and dose-dependent manner.

Most studies have reported that hypericin-induced cell killing can be shifted from apoptosis to necrosis by increasing the concentration of hypericin applied to the several cell lines. The cellular response in HeLa cell line to 125 nM photoactivated hypericin was determined as apoptotic. In the same cell line, increasing the hypericin concentration to 1 μM resulted in necrosis [12]. Apoptotic effects of hypericin were evaluated in HL-60 leukemic cell line by Lee et al. [13], who showed that treatment with hypericin at a concentration of 0.15 to 0.2 μM induced both differentiation and apoptosis. In another experience obtained from a study designed with human nasopharyngeal carcinoma, colon and bladder cells by Ali et al. [14], it was shown that with high hypericin dose (> 5 μM), electron microscopy revealed damage to plasma membrane indicating a mechanism for necrosis, whereas sublethal doses of hypericin (< 2.5 μM) resulted in induction of apoptosis after photodynamic therapy. The cytotoxic effects of hypericin on HL-60 leukemic cells in this study were revealed by using trypan blue dye exclusion test and XTT assay. We found the IC_{50} dose of hypericin to be 500 nM and this dose value showed a parallel cytotoxicity with the 1/10.000 dilution of *H. perforatum* extract after 72 hour of incubation.

Several mechanisms are attributed to the apoptotic effects of hypericin. In many studies, it has been shown that hypericin photosensitization inhibits protein kinase C [15]. The other possible steps taken up in apoptotic mechanisms are changes in the caspases, loss of mitochondrial transmembrane potential, and release of cytochrome C from mitochondria [16-18]. In this study, beginning from 48 hours, and especially in 72 hours, IC_{50} dose (500 nM) of hypericin exhibited an apoptotic effect in HL-60 leukemic cells. We also found that hypericin inhibited the expression of telomerase by decreasing h-TERT level.

It has been shown that h-TERT, which is a catalytic subunit of telomerase, is associated with telomerase activity [19,20]. In this study, h-TERT ratio of the incubated HL-60 leukemic cells with hypericin decreased after treatment with 500 nM dose for 48 hours. It is well known that progressive telomere shortening to a critical level results in senescence of normal cells by inducing apoptosis [21].

In conclusion, no study in the literature to date has been reported regarding the effect of hypericin on h-TERT activity. Therefore, this study may contribute to the literature about the mechanisms of hypericin-induced apoptosis.

Until this study, there also has been no comparable published study about the cytotoxic effects of *H. perforatum* extract and hypericin in the same conditions. We found that both the plant extract and hypericin showed cytotoxic effects in a dose-and time-dependent manner. In addition, hypericin showed apoptotic effects by reducing h-TERT activity. These results would support the use of *H. perforatum* L. in chemo-prevention of cancer and open new windows for the potential use of hypericin either as a single agent or in combination with the currently available anti-cancer agents.

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