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Research Article
Proapoptotic Effect of *Hypericum perforatum* (St. John's Wort) Extract in Human Colorectal Adenocarcinoma Cell Line HT29

[1]Arash A. Yegani, [2]Erman S. Istifli, [1]Ibrahim O. Tekeli, [1]Fatih Sakin and [3]Halil M. Kaplan

[1]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Mustafa Kemal University, 31040 Antakya, Hatay, Turkey
[2]Department of Biology, Faculty of Science and Letter, Cukurova University, 01380 Adana, Turkey
[3]Department of Pharmacology, Faculty of Medicine, Cukurova University, 01380 Adana, Turkey

Abstract

Background and Objective: Hypericum perforatum (HP) is known to have therapeutic effects in many types of cancer. Although the active substances hypericin and hyperforin in the composition of HP have been shown to exert apoptotic effects in cancer cell lines, research into the anticancer effects of the total extract of HP is meagre. The aim of this study was to investigate the proapoptotic effect of HP on human colorectal adenocarcinoma cell line HT29. **Materials and Methods:** HT29 cells (5×10^4 cells cm^{-2}) were exposed to 99.2% medium and pharmacologically effective concentration of HP extract (0.8%) for 48 h. Cells were then washed in phosphate buffered saline PBS and lysed in RIPA buffer for 15 min on ice followed by centrifugation at 15000 rpm for 20 min and supernatants were taken and pellets were discarded. ELISA test was used to examine the expression and activity of cleaved caspase-3, Bax, Bcl-2, Wee1, GADD153, GRP78 and AIF proteins in HT29 cells. **Results:** ELISA test results indicated that the activities of proapoptotic proteins Bax, cleaved-caspase 3, Wee1, GRP78, GADD153 and AIF in human HT29 cell line were significantly increased in 48 h treatment with HP extract at 0.8% concentration. On the other hand, the activity of Bcl-2 was observed to decrease significantly. **Conclusion:** The results implicated that the HP extract in its original form effectively induces proapoptotic alterations in HT29 cells and a possible mutual signaling between the proapoptotic Wee1, GRP78, GADD153, AIF, Bax and cleaved caspase-3 proteins can contribute to this effect.

Key words: *H. perforatum*, HT29 cells, colorectal cancer, apoptosis, antimutagenic activity, proapoptotic effect, phytochemical

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Corresponding Author: Halil M. Kaplan, Department of Pharmacology, Faculty of Medicine, Cukurova University, 01380 Adana, Turkey
Tel: +90 3223386084/123 Fax: +90 3223386070

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

INTRODUCTION

In recent years, interest in plant-derived extracts and molecules has increased in the treatment of various diseases. In this context, *Hypericum perforatum* (HP) has been the most frequently studied plant in the Hypericum genus. HP is a flowering plant used as a natural remedy in the treatment of different diseases for centuries[1]. HP extract contains hypericin, hyperforin, pseudohypericin, flavonoids, naphtodianthrones, phloroglucinols, procyanidins and essential oils that show biological activity within the cell[2,3]. HP extract is thought to act on multiple targets within the cell at low concentrations and has been reported to block metastasis as well as its antidepressant, antibacterial and cytotoxic effects[4,5]. The basis of these effects have been thought to be due to the hypericin component found in the extract which has an effect on various key intracellular pathways[6].

In OECD (Organisation for Economic Co-operation and Development) countries, colorectal cancer is the third most common cancer type after prostate and lung cancer in men and the second most common cancer type in women after breast cancer[7-9]. Therefore, plant-based alternative treatment approaches with low or no side effects in the treatment of colorectal cancer are of high importance.

Pre-treatment with HP was shown to exert antimutagenic activity against chromosome breaking effects of cyclophosphamide in wistar rat bone marrow cells, however, HP treatment alone did not lead to cytotoxic activity in treated healthy rats when compared to control group[10]. It has been reported that photoactivated-hypericin induced cell death in CCL-220.1 cell line via caspase-3 activation and apoptosis[11]. Hyperforin and hypericin, the individual constituents of HP, have been shown to cooperatively induce toxicity in leukemia cell lines K-562 and U-937 by a caspase-3 dependent mechanism and total cytotoxicity of HP extract was considered more important than the toxicity of single compounds[3]. More recently, a study showed that both hypericin and hyperforin rich HP extracts inhibited HT29 cell growth and that the efficacy of the whole plant extracts in inducing apoptosis was greater than the purified hypericin and hyperforin[12]. In another study, it was reported that hypericin did not inhibit cell division in mouse breast cancer cell line as potent as the HP whole extract did and therefore the study concluded that hypericin was not the only compound with anti-cancer properties in HP[13]. Similarly, in previous studies carried out by different investigators, whole plant extracts were shown to be more effective in inducing cytotoxicity in cancer cells both *in vitro* and *in vivo*[14,15].

Therefore, in this study, the effect of HP whole plant extract on apoptotic modulator proteins was investigated. For this purpose, proapoptotic (Bax, caspase-3, AIF), antiapoptotic (Bcl-2), mitotic growth-arrest proteins (Wee 1, GADD153) as well as the dual-function GRP78 protein levels in HT29 cell line treated with HP extract were assessed for the first time. A direct enzyme-linked immunosorbent assay (direct ELISA) was used to determine the protein levels. The inducing effect of individual bioactive compounds in HP on apoptotic proteins have long been known in the literature. In this context, the present study is the first to show the effects of a total extract of HP on Bax, caspase-3, AIF, Bcl-2, Wee 1, GADD153 and GRP78 proteins.

MATERIALS AND METHODS

This study was carried out in the department of Pharmacology, Faculty of Medicine, Çukurova University between the dates of 15-01-2018 to 30-07-2018.

Chemicals: McCoy's 5A Medium, RIPA buffer, fetal bovine serum, PBS, NaCl, TritonX-100, EGTA, dithiothreitol, NaF, Tris-HCl, Na_3VO_4 were obtained from Sigma-Aldrich, Inc. All the ELISA kit were purchased from Shanghai Sunred Biological Technology Co., Ltd. In addition, the Bradford dye reagent were purchased from Bio-Rad Laboratories, Inc.

Plant extraction: *Hypericum perforatum* (HP) L. was grown in Pozant Province in Turkey and was harvested in August. The aboveground parts (leaves, flowers and stem) were dried and then mixed with 80% ethanol (v/v) and incubated overnight with shaker. After this period, extract was filtered using a filter cartridge and ethyl alcohol was evaporated *in vacuo* at 70°C with minor modifications as described previously[16].

Cell culture: Human HT29 cell line was obtained from American Type Culture Collection (ATCC). Basal cancer cells were grown on modified McCoy's 5A Medium (10% fetal bovine serum). The cells were incubated in a humidified atmosphere at 37°C in 5% CO_2[17].

Cell homogenization: The pharmacologically effective concentration of HP (0.8%) was selected according to the study of Ferguson *et al.*[13]. Cells (5×10^4 cells cm^{-2}) were exposed to 99.2% medium and 0.8% HP extract for 48 h. Cells were then washed in PBS and lysed in RIPA buffer (150 mmol L^{-1} NaCl, 0.5% TritonX-100, 20 mmol L^{-1} EGTA,

1 mmol L^{-1} dithiothreitol, 25 mmol L^{-1} NaF, 50 mmol L^{-1} Tris-HCl [pH 7.4], 1 mmol L^{-1} Na_3VO_4) for 15 min on ice followed by centrifugation at 15000 rpm for 20 min and supernatants were taken and pellets were discarded.

Total protein quantification: Bradford method was used to quantify the total protein in homogenized cells[18]. Using bovine serum albumin (1 μg mL^{-1}), 1, 2, 3, 5, 7, 8, 10 (μg mL^{-1}) of standards were established. The 10 μL was taken from each sample and completed to 100 μL by adding distilled water. Lastly, after adding 1 mL Bradford solution to standards and samples, eppendorf tubes were vortexed and the absorbance at 595 nm were measured manually. Protein quantification (μg μL^{-1}) was performed according to the standard curve drawn in Prism software.

ELISA (enzyme linked immunosorbent assay) test: Direct ELISA test was used to examine the expression and activity of cleaved caspase-3, Bax, Bcl-2, Wee 1, GADD153, GRP78 and AIF proteins.

Statistical analysis: Parameters of control and HP-treated groups were compared by unpaired Student's t-test. Data is showed as mean±SEM. Difference at $p<0.05$ was considered as significant.

RESULTS

In this study, HT29 cells were exposed to a total extract of *Hypericum perforatum* (HP) at a concentration of 0.8% (99.2% medium, 0.8% HP) for 48 h. The control group was consisted of untreated HT29 cells.

HP treatment significantly rised the levels of the Bax (Fig. 1a) and cleaved-caspase 3 (Fig. 1b) in HT29 cells when compared with the control group ($p<0.05$).

On the other hand, HP treatment significantly reduced levels of the antiapoptotic Bcl-2 (Fig. 1c) in HT29 cells when compared with the control group ($p<0.05$).

In HT29 cells treated with HP, the levels of AIF (a proapoptotic protein), levels of Wee1 and GADD153 (proapoptotic mitotic growth-arrest proteins) as well as the levels of GRP78 protein that serves as an apoptotic marker on the surface of cancer cells were also investigated. Table 1 demonstrates the results of HP treatments on the levels of Wee1, AIF, GADD153 and GRP78 proteins in human HT29 cell line. Treatment with HP resulted in statistically significantly increased levels of AIF, Wee1, GADD153 and GRP78 in HT29 cells when compared to control ($p<0.05$) (Table 1).

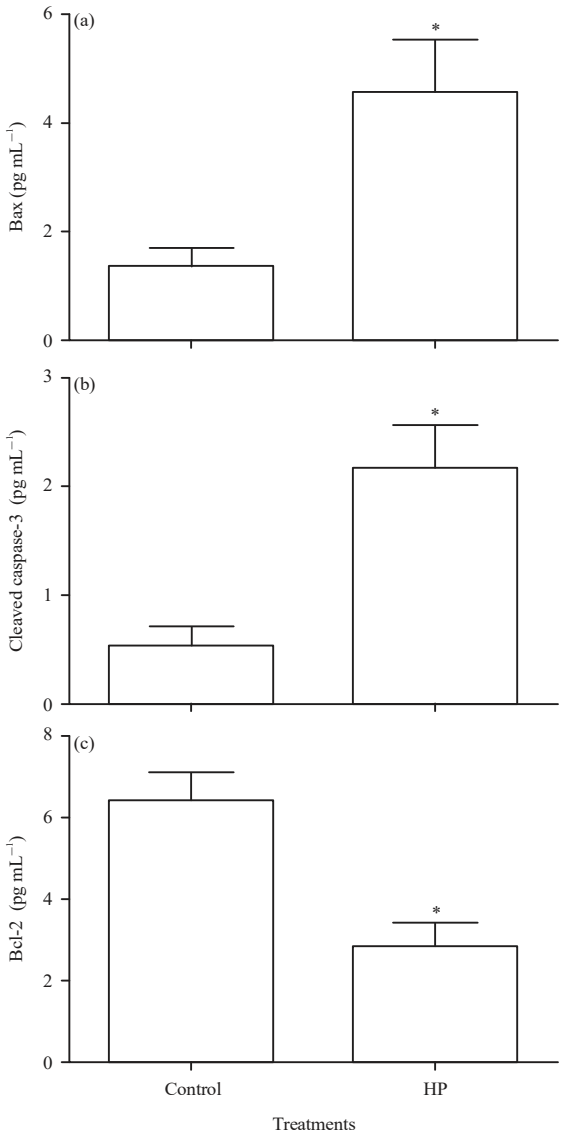

Fig. 1(a-c): Effect of extract treatment on (a) Bax expression, (b) Caspase-3 activity and (c) Bcl-2 expression

Data are presented as Mean±SEM, differences between parameters of control and extract treated group were analyzed unpaired Student's t test, *Difference between control and extract treated group is significant with $p<0.05$

Table 1: Effects of HP on expression of mitotic division inhibitors (Wee1 and GADD153), proapoptotic AIF and dual function GRP78 proteins

Proteins	Control (pg mL^{-1})	Extract (pg mL^{-1})
Wee 1	0.15±0.02	1.10±0.022*
AIF	0.68±0.015	1.10±0.09*
GADD153	0.18±0.023	1.68±0.25*
GRP78	0.43±0.02	1.32±0.18*

Results are presented as Mean±SE, statistical analysis: Student t-test, *Control $p<0.05$

DISCUSSION

In this study, *Hypericum perforatum* (HP) extract significantly increased the concentration of proapoptotic proteins (Bax, Caspase 3 and AIF), mitotic-growth arrest proteins (Wee 1 and GADD153) and dual function protein GRP78 while significantly reducing the amount of Bcl-2, an antiapoptotic protein in human colorectal adenocarcinoma cell line HT29. In addition, current study demonstrated that HP total extract is as effective as individual bioactive components, hypericin and hyperforin, to induce apoptosis. Therefore, findings of present study confirmed that HP total extract may be an alternative approach in cancer treatment compared to conventional chemotherapy.

In this study, the amount of all the proteins that were tested for their expression (except Bcl-2, an antiapoptotic protein) increased at least 2-fold compared to the control (Fig. 1a, b and Table 1). A 2-fold increase compared to the control is a statistically significant increase and therefore these results showed that HP exerts a strong apoptotic modulatory effect in HT29 cells.

Bax protein is a member of the Bcl-2 family and shows a structural peculiarity that promotes apoptosis[19]. The ratio of Bax to Bcl-2 determines the sensitivity of a cell to apoptosis[19,20]. On the other hand, cleaved caspase-3 is known to act downstream of Bax/Bcl-2 control and plays an important role in the execution of apoptosis[21]. In the present study, treatment of HT29 cells with HP extract significantly increased the levels of Bax and cleaved caspase-3 levels, whereas it significantly reduced the levels of Bcl-2. Similarly to the results obtained in this study, Cinci *et al*.[2] reported that HT extract significantly increased the caspase-3 activity in HT29 cells compared to control. Furthermore, Merhi *et al*.[22] reported that hypericin (photoactivated hypericin), a component of HP extract, increased the Bax and caspase-3/7 activity in HT29 adenocarcinoma cells in 1, 3, 6 and 24 h treatment. Hyperforin, another constituent of HP, was shown to increase apoptosis by activation of caspase-3 and inhibition of Bcl-2 protein expression in human myeloid cancer cells. Moreover, it has been shown that proliferation of CRL2539 cells (mouse breast cancer cell line) was inhibited in a dose-dependent manner by application of HP extract[13].

One of the other proteins involved in the apoptotic pathway independent of caspases is the apoptosis inducing factor, AIF. To the best of our knowledge, the present study is the first to show the increase in the expression of AIF protein in HT29 cells after HP treatment. It is interesting to note that although photo activated hypericin has been shown to increase AIF expression in HT29 cells[23], this study showed that the same effect was also produced using the whole extract of HP. Therefore, it was considered that HP extract has the same capacity in the effective stimulation of apoptosis in cancer cells. In addition, since AIF plays a role in apoptosis stimulation by caspase-independent pathways, it can be said that HP may induce cell death through different pathways independent of caspases. In support to this hypothesis, it has been reported that hypericin-mediated photodynamic therapy triggers autophagy by oxidative damage to Ca^{2+}-ATPase (SERCA) pumps of sarco (endo) plasmic reticulum in HeLa cells[24]. Therefore, since hypericin is a component of HP, it can be said that the same pathway of death can be triggered by HP, the compound tested in this study.

Although the cytotoxic effect of HP on healthy cells was not investigated in this study, it has been shown that HP had no significant cytotoxic effect on healthy eukaryotic cells (*Allium cepa* meristematic cells) and its effect on cell division is reversible[10]. In the present study, on the other hand, HP extracts were found to significantly increase the expression of Wee1 and GADD153 proteins, inhibitors of mitotic division, in HT29 cell line at a concentration of 0.8% in 48 h treatment. There have been no research in the literature on the effects of HP extract on Wee1 and GADD153 gene expression. However, the results obtained in the current study demonstrate for the first time that the increase in Wee1 and GADD153 protein expression leads to genome-wide blockage of mitosis and apoptotic signals may be produced by the genome. Therefore, inhibition of mitotic division by HP in cancer cells indicates that this plant may be beneficial in the treatment of cancer.

In this study, the HP extract significantly increased the concentration of GRP78 protein in HT29 cells. The GRP78, which belongs to heat shock protein 70 family, is a chaperone that functions in the folding, maturation and transport of polypeptides and proteins in the endoplasmic reticulum[25]. The elevated GRP78 protein level on the tumor cell surface has been shown to be associated with decreased tumorigenesis, tumor growth and increased apoptosis[26]. Although GRP78 does not play a direct role in apoptosis, it plays a role as a flag in the orientation of cancer cells to apoptosis and cancer cells can be successfully killed with ligands (cytotoxic agents) that exhibit[27] high affinity to GRP78. In addition, a breast cancer study showed a significant correlation between the over-expression of GADD153 and increased cell surface[28] GRP78. Thus, the results obtained from the present study indicate an increased possibility of a mutual signal trafficking between Wee1, GADD153, AIF and GRP78 in the apoptosis signaling pathway.

CONCLUSION

In this study, it has been found that the HP extract significantly increased proapoptotic protein expression in HT29 cancer cell line, while significantly reducing antiapoptotic protein (Bcl-2) expression. Although the current study is an initial attempt to demonstrate the effect of HP on the expression of these proteins, it clearly demonstrated the apoptosis-inducing effect of HP on human HT29 cells and suggested that HP may be a promising plant in cancer treatment. However, in the literature, there are no mechanistic research on HP's molecular-level interaction with apoptotic proteins. Therefore, it has been envisioned that future studies on the anti-cancer effect of HP should focus on the elucidation of binding modes with receptor proteins.

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SIGNIFICANCE STATEMENT

This study discovered the apoptosis-inducing effect of the total extract of *Hypericum perforatum* in human colorectal adenocarcinoma cancer cell line HT29 and also showed that *Hypericum perforatum* has a positive effect on the expression of previously unexamined apoptotic modulatory proteins Wee1, GADD153, AIF and GRP78. This study will help the researchers to uncover the importance of alternative plant-based remedy based on *Hypericum perforatum* in the treatment of human colorectal cancer that many researchers were not able to explore. Thus, a new theory that the total extract of *Hypericum perforatum* alone is effective and sufficient in the fight against human colorectal cancer versus conventional chemotherapy may be arrived at.

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