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HYPERICUM PERFORATUM L.: AN OVERVIEW OF THE ANTICANCER POTENCIES OF THE SPECIMENS COLLECTED FROM DIFFERENT ECOLOGICAL ENVIRONMENTS

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Abstract

Hypericum genus holds an important place in the flora of Turkey with 46 endemic species out of its naturally growing 96 species. *H. perforatum* is the most popular and common species. This species has been traditionally used in pain control, wound healing and especially as antidepressant. Its anticancer effects have been lately attracting much attention. To investigate and compare the antiproliferative effects of extracts of the samples collected from eleven different "States" of Turkey. The antiproliferative effect of extracts was determined by WST-1 method on HeLa, U2OS, HCC-1937 cancer cells and MRC-5 non-cancer fibroblast cells. Furthermore, cell death mechanism of the extracts was analyzed by investigating the cleavage level of caspase-7 and PARP-1, which are hallmarks of apoptosis and also the levels of LC3-II and p62 protein levels as markers of autophagy. Only HP10 (collected from Çankırı-Ilgaz) was found to induce apoptosis both using immunoblotting and flow cytometry analysis, while almost all of the extracts cause the induction of autophagy in HeLa cells at their IC_{50} values. HP10 coded sample was collected from the transition zone between the Black Sea and Central Anatolian Regions, the collection area with highest altitude. According to our results different climatic features effect plant contents as well as their therapeutic effects. Moreover, the amount of compounds that have anticancer activity in the plant undergo a change with altitude.

Key words: Antiproliferative effects, Apoptosis, Autophagy, Cancer, Cytotoxicity, *Hypericum perforatum*.

Introduction

H. perforatum is a plant which has been used for its medicinal effects since Greek and Roman times. It is a perennial herbaceous plant belonging to the family Hypericaceae. The members of this family occur as herbs, shrubs, and infrequently trees, and are found in a variety of habitats in temperate regions and in high mountains in the tropics, avoiding only zones of extreme aridity, temperature and/or salinity. There are 482 *Hypericum* species distributed in different parts of the world from the equator to the Nordic countries (Crockett & Robson, 2011; Nürka & Crocket, 2011, Meseguer *et al*., 2013; Abrahamson & Kloet, 2019). Turkey is an important center of this genus. A total of 46 taxa out of 96 are endemics to Turkey (Güner *et al*., 2012).

Galen first explained its medicinal characteristics in the 2nd century. Traditionally, it has been used orally in the treatment of excitability, neuralgia, sciatica, menopause neurosis, anxiety/depression and externally in the wound treatment in the form of infusion, decoction and maceration (Barnes *et al*., 2001; Brunakova & Cellarova, 2016; Marrelli *et al*., 2016). The plant is also classified by the Council of Europe as a natural flavor source with limits. *H. perforatum* is among the bestselling dietary supplements in the world markets (Barnes *et al*., 2001; Ernst, 2019). The use of this plant especially in the treatment of depression, AIDS and ulcers has increased the sales figures at global level.

H. perforatum is commonly known as St. Johns worth. There are transparent pores on the leaves that can be seen when exposed to light. The name of the plant originates from these pores because perforatum means porous in Latin (Dauncey *et al*., 2019). When rubbing the black glands on the edges of the leaves of the plants and flowers, a red liquid is released. This red liquid is due to one of its main components hypericin (Çırak & Kurt, 2014). The other major ingredient is hyperforin. These two main substances are responsible for different effects of this plant (Percifield *et al*., 2007; Kimakova *et al*., 2018). It is also rich in flavonoids, tannins and phenolic substances (Nahrstedt & Butterweck, 1997; Nigutova *et al*., 2019).

Hypericin is a substance having the structure of naphthodianthrone from the anthraquinone derivatives. Other ingredients in this structure are pseudohypericin, isohypericin, protohypericin and protopseudohypericin. Protohypericin and protopseudohypericin are biosynthetic precursors of hypericin and pseudohypericin. They are sometimes referred to as 'total hypericins' (Barnes *et al*., 2001) (Fig. 1). This compound is one of the most powerful photosensitizer substances found in nature, therefore important as a non-toxic photosensitive drug in photodynamic therapy (PDT) and photodynamic diagnosis (PDD) as well as its antidepressant effect (Jendzelovska *et al*., 2016; Majernik *et al*., 2019). Studies have shown that neoplastic tissues are more sensitive to hypericin than normal cells. For this reason, hypericin can be used as a marker for photodynamic diagnosis for tumor detection and visualization (Karioti & Bilia, 2010). In addition, Hypericin-PDT causes tumor cell death by various mechanisms including cell death due to apoptosis, necrosis and autophagy (Kleemann *et al*., 2014). The effect of hypericin has been investigated by *in vitro* studies using various cancer cells (human bladder cancer cells, human glioma cancer cells U-87, human mucosal carcinoma cells, pediatric hepatocellular carcinoma HepG2 cells hepatic hepatoblastoma cells, epidermoid carcinoma cells (A431), human umbilical endothelial cells, MDA231 human mammary carcinoma cells, human lung SpcA1 cancer cells and rhabdomyosarcoma cells).

All have proved that it has positive cytotoxic effect (Ali *et al*., 2001). Hypericin-PDT is also seen as a potential adjuvant to therapy for melanoma (Sharma & Davids, 2012). In a study investigating the effects of hypericin application topically in the treatment of squamous cell carcinoma, a new epithelium formation was observed on the surface of malignancy without penetrating necrosis or cell loss in surrounding tissues (Alecu *et al*., 1998). In addition, hyperisin has been reported to be a component of the inducible plant defense response so it is also a component of the plant's own defense mechanism (Çirak *et al*., 2005).

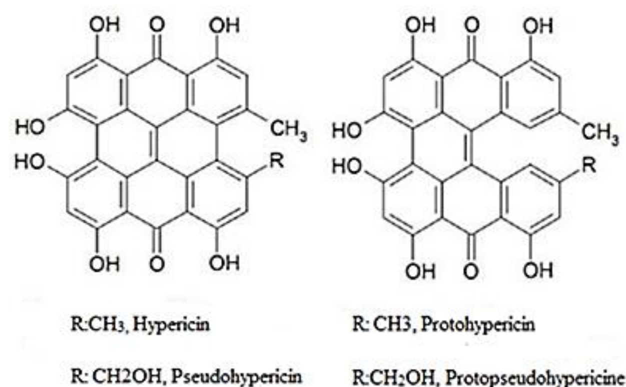

Fig. 1. Chemical structures of hypericin, protohypericin, pseudohypericin and protopseudohypericin.

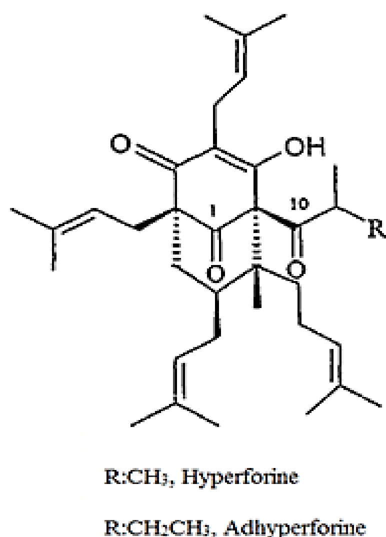

Fig. 2. Chemical structures of hyperforine and adhyperforine.

The phloroglucinol-derived hyperforin and adhyperforin are the major bioactive molecules of *H. perforatum* (Fig. 2). Hyperforine is a highly lipophilic, temperature sensitive, unstable compound, but sensitive to photodegradation. Hyperforin and adhyperforin in solution show similar stability profiles, therefore, it has taken a long time to explain their pharmacological effects. Recently, hyperforin has emerged as a compound of interest, with several activities such as anti-inflammatory, anti-depressive, anti-bacterial, antituberculosis and anti-fungal (Albert *et al*., 2002; Berköz *et al*., 2018). It has also been observed to show neuroprotective effects against Alzheimer's disease neuropathology (Griffith *et al*., 2010). The anti-cancer effects of hyperforin have become increasingly important. The antitumor and proapoptotic effects against certain cancer cell lines have been demonstrated in several studies. Hyperforin has been shown to inhibit leukemia K562 and U937 cells, glioblastoma LN229 cells and B cell chronic lymphocytic leukemia cells (Quiney *et al*., 2007; Hostanska *et al*., 2003). It has been suggested that leukemia cell K562 and U937 lines are more susceptible to hyperforine and are therefore partly responsible for the cytotoxic effect. Similarly, it shows antitumoral activity on breast cancer cells by inducing apoptosis and inhibiting angiogenesis (Liu *et al*., 2007). It has been found that, in in vivo treatment with hyperforin Kaposi's sarcoma (a highly angiogenic tumor), both growth and vascularization are reduced (Lorusso *et al*., 2009).

Flavonoids in *H. perforatum* include flavonols (kaempferol, quercetin), flavones (luteolin), glycosides (hyperside, isoquercitrin, and rutin), biflavones (biapigenin), amentoflavone, myricetin, hyperin, oligomeric proanthocyanadins, and miquelianin. All of these are biogenetically related. They are secondary metabolites with phenolic structure found in many plant species. *H. perforatum* is a rich source of flavonoids. The flavonoids and their derivatives; isolated from *Hypericum* species; exhibit potent anti-inflammatory, antioxidant, anti-ulcer and anti-depressant (Barnes *et al*., 2001; Tatsis *et al*., 2007); anti-fungal, anti-microbial and anti-cancer activities (Carlo *et al*., 2001; Greeson *et al*., 2001). Imbalance in the level of free radicals has a mutagenic or carcinogenic effect leading to cancer development, therefore dietary supplements with antioxidant effects play an important role in the prevention and improvement of various types of cancers. Phenolic compounds, especially flavonoids, have long been considered chemopreventive agents in cancer treatment because of their high antioxidant activity. However, many scientific researches have shown that various flavonoids can promote apoptosis in various cancer cells (Brusselmans *et al*., 2003, 2005). In particular, quercetin, one of the flavonol members found in *H. perforatum* is reported as an interesting anticancer agent against prostate and breast cancers (Brusselmans *et al*., 2005; Kumar & Pandey, 2013). The effects of quercetin have been tested on other cancer cell lines, prostate adenocarcinoma LNCaP cells, pheocromocytoma PC-12 cells, colon carcinoma CT-26 cells, acute lymphoblastic leukemia MOLT-4 T-cells, human prostate PC-3 cells estrogen receptor-positive breast cancer MCF-7 cells, human myeloma U266B1 cells, ovarian cancer CHO cells and human lymphoid Raji cells and results have shown that quercetin can significantly induce apoptosis of each tested cell line compared with the control group (Hashemzaei *et al*., 2017).

Rutin is a glycoside of the flavonoid quercetin. It has significant and potentially beneficial effects in reducing the amount of precancerous lesions and inducing apoptosis in large bowel cancer (Volate *et al*., 2005). The routine causes of proliferation and induction of apoptosis in human neuroblastoma LAN-5 cells have also been investigated and the results have revealed that routine produces significant antineuroblastoma effects via induced G2/M uptake and induced cell apoptosis as well as regulating apoptosis-related gene expression (Chen *et al*., 2013).

Kaempferol is another flavonoid derivative with anticancer activity. Studies have shown that it exhibits significant anticancer activity against A375 cells. Effect of kaempferol is reported to be due to induction of apoptosis in A375 cells of human malignant it has also been demonstrated that it has an ability to induce G2/M cell cycle arrest and inhibit the cell migration potential of A375 cells (Yang *et al*., 2018). Kaempferol has also been reported to effectively inhibit migration activity of human pancreatic cancer cells at relatively low doses without toxicity.

Herbal treatment is one of the most commonly used alternative treatment method (Ozturk & Hakeem, 2018, 2019 a,b) in different health problems. In view of this, nowadays anti-cancer effects of many medicinal plants are investigated, proven and made available. In general, the medicinal plants constitute the raw material of many FDA-approved anti-cancer semi-synthetic drugs (Mishra & Tiwari, 2011). In a study investigating anti-cancer drugs between 1930 and 2014, it has been mentioned that 48 percent of anticancer drugs are natural products and derivatives (Newman & Giddings, 2014; Newman & Cragg, 2016). Anti-cancer effects of *Hypericum* species have been investigated recently in depth and interesting results have been recorded. These studies have shown that anti-cancer activity of this plant generally occurs in two different ways. The first depends on its photosensitizing effect and the second is due to its chemo preventive effect. The plant has anti-proliferative and apoptosis inducing effects on various cancer cell lines.

Some studies have been carried out on the anticancer effects of *H. perforatum* extracts. Part of the anticancer effect is due to the photosensitizing effect of the plant. The extracts of this species are promising photosensitizers in clinical photodynamic therapy due to their excellent photosensitivity and tumoritropic properties. It also causes tumor cell death by various mechanisms including extract-induced cytotoxicity, apoptosis, necrosis and autophagy. In one of the studies *Hypericum* extracts have proved effective in killing both A375 and 501ml pigmented as well as UCT Mel-1 pigmented melanoma cells by specific mechanisms including the externalization of phosphatidylserines, cell contraction and loss of cell membrane integrity (Kleemann *et al*., 2014). In another study, extracts from *H. perforatum* flowers characterized by spectroscopic profile and hypericin contents have been tested for growth and apoptotic death of K562 cells, a human eritroleukemic cell line, the extracts were able to reduce growth of K562 cells and induce different degrees of apoptosis kinetics. In a study on the standardized extracts, some activity losses due to variables have been recorded showing that ingredients other than hyperisin do contribute to anti-cancer effect (Valletta *et al*., 2018). *H. perforatum* methanol extract and pure hypericin were tested comparatively by following the development of human erythroleukemic cell line. The data has revealed that purified hypericin has only a weak inhibitory effect on cell growth and no effect in inducing apoptotic cell death. In contrast, *Hypericum* flower extract has significantly inhibited the cell growth and induced apoptotic cell death, theirby confirming the interesting role of *H. perforatum* in cancer treatment and stressing the fact that there are effect of agents other than hypericin (Roscetti *et al*., 2004). Hypericins (hypericin, pseudohipericin, etc.); as the main active substance; have been identified only in the methanolic (lipophobic) extract of this herb and not in the active lipophilic extract, latter extract stimulated apoptosis at low concentrations and inhibited T24 and NBT-II urinary bladder tumor cells growth. The results further enlightened that lipophilic extract of *H.perforatum* has an interesting new antiproliferative potential against bladder cancer (Skalkos *et al*., 2005). In another study effects of extracts prepared from fresh and dried *H. perforatum* plants have been investigated on K562, U937, LN229 glioblastoma cell lines and normal human astrocytes in the dark and after exposure to 7.5 J/cm^2 white light as compared to hypericin. The data reported suggests that illumination is not essential for the growth inhibitory and apoptotic effects of *H. perforatum* extracts, but light activation potentiates the same. In addition, hyperforin is thought to be partly responsible for these effects in the dark (Hostanska *et al*., 2002).

In this study, infusions and ethanol extracts from *H. perforatum* samples collected from eleven different areas in Turkey were used. The cytotoxic effects of samples on cancer and healthy cells were also examined cytotoxicity of infusions and ethanol extracts were investigated with HCC1937, MCF7, HeLa as tumor cell lines, and MRC5 as non-tumor cells. The mechanisms of extracts showing cytotoxic effect on cancer cells were examined using apoptosis and autophagy markers.

Materials and Methods

Plant samples: *H. perforatum* plants were collected during flowering season from different locations in Turkey covering the geographical regions like Marmara, Aegean and the Black Sea. The identification of the plants was done by Dr. S. Türkseven from Ege University, Faculty of Agriculture Department of Field Crops. Sample codes together with the locations from where the samples were collected, altitude and habitats are presented in Table 1. Plants were dried outdoors at room temperature and dried samples powdered using mill with a 0.2 mm diameter. Powdered material (10 g) was extracted by shaking with maceration for 24 hours after adding 200 ml of ethanol. The extracts were concentrated to dryness under a vacuum at 40ºC by using a rotary evaporator and stored at +4ºC in the dark until used. 2 percent infusions were prepared from all collected samples, filtered and concentrated to dryness under vacuum at 80ºC by using a rotary evaporator and stored at +4ºC in the dark until use.

Cytotoxicity assay: Human cervical cancer cell line (HeLa), human breast cancer cell line (HCC-1937), human osteosarcoma cell line (U2OS) and, human lung fibroblast cell line (MRC-5) were obtained from American Tissue Culture Collection (ATCC). 7500 cells were seed on the 96 well dishes and treated with increased doses of extracts (0, 5, 10, 20, 30, 40, 50, and 75 µg/ml) for 48 hours. After treatment cell viability was assessed by WST-1 reagent (ROCHE) following the instruction of the manufacturer. IC_{50} values of extracts were determined using a sigmoid-dose response curve and nonlinear regression. Data are presented as means ± Standard Deviation (SD).

Table 1. *H. perforatum* sample codes, collected area, altitude and habitats.

Code	City	Location	Altitude	Habitats
HP01	Tekirdağ	Yenice	222 m	Wheat field
HP02	Tekirdağ	Center	105 m	Wheat field
HP03	Edirne	Keşan Paşayiğit	115 m	Wheat field
HP04	Edirne	Uzun Köprü	96 m	Roadside
HP05	Çanakkale	Korudağ	238 m	Forest
HP06	Çanakkale	Gelibolu	30 m	Roadside
HP07	Bursa	Gemlik	137 m	Roadside
HP08	Sakarya	Düzce	33 m	Roadside
HP09	Bolu	Bolu Mount	720 m	Forest
HP10	Çankırı	Ilgaz	844 m	Roadside
HP11	Samsun	Hacılı Passageway	688 m	Wheat field

Table 2. Cytotoxic potencies of *H. perforatum* L. ethanol extracts against HeLa, HCC-1937, U2OS, and MRC-5 cell lines; (IC_{50} µg/ml).

Code	HeLa	HCC-1937	U2OS	MRC-5
HP1	35.79 ± 0.38	42.88 ± 1.16	> 50	> 50
HP2	14.48 ± 1.15	8.14 ± 0.54	9.174 ± 1.17	10.70 ± 0.31
HP3	31.78 ± 1.09	15.40 ± 1.22	24.31 ± 0.79	21.83 ± 1.25
HP4	32.74 ± 0.94	30.99 ± 2.08	> 50	> 50
HP5	34.98 ± 1.53	37.18 ± 1.03	> 50	> 50
HP6	23.78 ± 1.37	33.85 ± 1.29	26.06 ± 0.98	33.95 ± 1.65
HP7	22.30 ± 1.60	36.08 ± 0.68	31.5 ± 1.10	38.55 ± 1.95
HP8	33.00 ± 1.30	> 50	> 50	> 50
HP9	27.66 ± 2.08	40.15 ± 0.46	> 50	> 50
HP10	23.73 ± 2.45	29.18 ± 3.09	26.53 ±3.52	39.5 ± 2.06
HP11	33.08 ± 3.12	41.25 ± 2.33	> 50	> 50

Immunoblotting: HeLa cells were treated with the IC_{50} concentrations of compounds or solvent control for 24 h and lysed with RIPA buffer (50 mm TRIS-HCl, pH 8.0, 1% NP-40, 0.1% SDS, 150mm NaCl, 0.1% Triton x-100, 5 mm EDTA) with protease inhibitors (ROCHE). Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Thermo Fisher). Equal amounts of proteins were loaded to the gels and proteins were separated by SDS-PAGE electrophoresis and transferred to PVDF membranes (Millipore). Membranes were blocked in PBS-0.1% tween-20 with 5% non-fat dry milk. After incubation with indicated primer antibodies (Cell Signalling) followed by secondary antibodies (Thermo Fisher Scientific), Chemiluminescence signals were detected using Clarity ECL substrate solution (BioRad) by Fusion-FX7 (Vilber Lourmat). Tubulin was used as loading control.

Flow cytometry: For apoptosis assay by flow cytometry, cells were seeded at 1×10^5 per ml on 6 well plates. Next day cells were treated with HP10 ethanol extract at its IC_{50} concentration for 24 hours. Cells were incubated with FITC-annexin V and 7-AAD (BD Biosciences) in binding buffer for 15 minutes in dark. Stained cells were immediately subjected to flow cytometry analyses using FACS Canto flow cytometer (BD Biosciences).

Results and Discussion

Cytotoxicity of extracts: The water and ethanol extracts of HP1-11 were evaluated for their cytotoxic activity against three cancer cell lines namely human cervical cancer cell line (HeLa), human breast cancer cell line (HCC-1937), human osteosarcoma cell line (U2OS) and one non-tumoral cell line, which is human lung fibroblast cell line (MRC-5).

While none of the water extracts exhibited cytotoxic effect at tested concentrations, most of ethanol extracts decreased cell viability with varied potencies the depending on cell type (Table 2). The extract of HP2 was found the be most potent one against HeLa, HCC-1937, U2OS and MRC-5 with IC_{50} values of 14.48±1.15, 8.14±0.54, 9.174±1.17, and 10.70±0.31 µg/ml, respectively, with no selectivity towards cancer and normal cells. HP3 ethanol extract inhibited cell proliferation of HCC-*1937* breast cancer cells with higher selectivity. HP1, HP4, HP5, HP9 and HP11 extracts exhibited similar cytotoxicity, where they showed cytotoxicity specifically against HeLa and HCC-1937 but did not effect the cell viability of U2OS and MRC5 cells at the tested concentrations. Ethanol extract of HP8 was cytotoxic only against HeLa cells. Lastly, HP6, HP7, and HP10 extracts showed cytotoxicity against all tested cell lines with different potencies (Table 2).

The effect of extracts on autophagy and apoptosis: Apoptosis is a programmed cell death mechanism of cells that are not needed and whose functions are impaired. The mechanism works without damaging the environment and without causing an inflammatory response, therefore death and construction continue in a dynamic balance to create tissue homeostasis (Wright *et al.*, 1996). Irregular apoptosis leads to neurodegenerative disorders, cancer and other hyperproliferative diseases. The effect is explained by several mechanisms, one of these is by caspases, an endoprotease family that controls inflammation and cell death. They are inactive in the cell but proteolytically activate each other. Their activation leads to the generation of a signal sequence permitting controlled destruction of cellular components. In the presence of apoptotic signal, initiator caspases (Caspase-8 and 9) are activated by dimerization and autocatalytic cleavage, whereas Caspase 2, 9 and 10 activate effector caspases (Caspase 3, 6 and 7) to inactivate enzymes necessary for DNA repair and replication, thus cutting the cell skeleton proteins and lead the cell membrane to budding (Spierings *et al.*, 2004). PARP-1 is a protein with various physiological and pathological functions and is one of the known substrates of caspases. The splitting of PARP-1 with caspases is considered to be a marker of apoptosis. Almost all caspases in vitro modify PARP-1 (Kaufmann *et al.*, 1993). Specific PARP-1 fragments help us understand the retention of cell death proteases and at the same time give information about different cell death types (Chaitanya *et al.*, 2010).

As an additional or alternative to apoptosis, autophagy is one of the cell death pathways, a lysosomal degradation of cellular proteins via autophagic vacuoles (Deretic, 2006). The latter unlike apoptosis provides protection of homeostasis by allowing the recycling of intracellular molecules in the case of cellular stress. It is triggered by cellular stresses such as infection, hypoxia, nutritional deficiency or reactive oxygen. A disorder in the autophagy system leads to some forms of cancer, some hereditary diseases, Alzheimer's disease and infections (Galluzzi *et al.*, 2012). On the other hand, modulating autophagy has become important as a promising therapeutic approach for some types of cancer (Bhat *et al.*, 2018). The protein called p62 is an ubiquitin-binding scaffold protein which breaks down with autophagy and allows binding of ubiquitin proteins to autophagy mechanisms for degradation in the lysosome. If autophagy is inhibited, the amount of p62 increases and decreases following its induction. Therefore, p62 protein is used as a marker to examine autofagic flux. Another marker is the microtubule chain-associated protein light chain 3 (LC3). The conversion of LC3I to LC3II is related to the number of autophagosomes therefore, LC3II formation indicates autophagy activation (Mizushima & Yoshimori, 2007). These markers allow to determine experimentally whether the specimens have anti-cancer effect. The effects of *Hypericum* species on apoptosis and autophagy are among the newly studied topics.

Since *H. perforatum* and/or its active secondary metobolites were indicated in autophagy and apoptosis, we first evaluated the effect of selected ethanol extracts at their IC_{50} values on the autophagy process in HeLa cells. We aimed to determine the levels of autophagy markers namely, LC3-I, LC3-II and p62 proteins via immunoblotting. An V-ATPase inhibitor Bafilomycin A1, a well-known autophagy inhibitor was used as positive control in the experiment (Mauvezin & Neufeld, 2015). Consistent with the previous reports autophagy inhibitor Baf A1 increased both the LC3-II and p62 protein levels indicating its autophagy inhibitory effect (Redmann *et al.*, 2017). While all tested compounds showed augmented LC3-I to LC3-II conversion levels compared to control, they decreased p62 protein levels (Fig. 3). This data suggests that the extracts of HP2, HP3, HP4, HP5, HP6, HP7, HP9, HP10, and HP11 induce autophagy process. Next, we tested the effect of these extracts on apoptosis by evaluating the well known apoptosis markers PARP-1 and caspase-7 by immunoblotting assay. We used staurosporin which is known to induce apoptosis and the extracts at their IC_{50} concentrations. While staurosporin showed induction of apoptosis as expected, only the extract of HP10 treatment enhanced the cleavage of PARP-1 and caspase-7 and induced apoptosis in HeLa cells (Fig. 4).

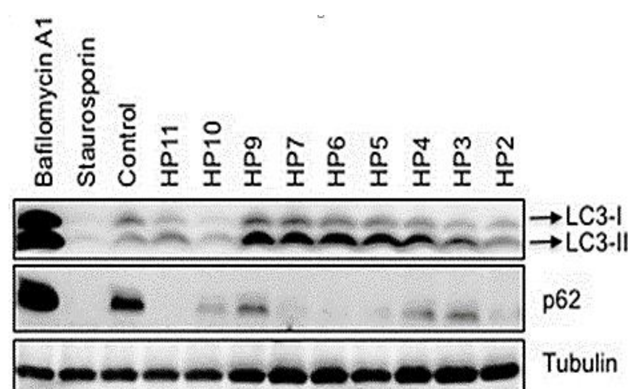

Fig. 3. The effect of extracts on autophagy marker proteins in HeLa cells.

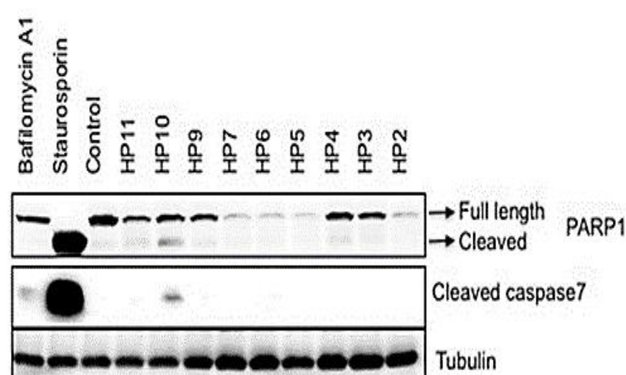

Fig. 4. The effect of extracts on apoptosis marker proteins in HeLa cells.

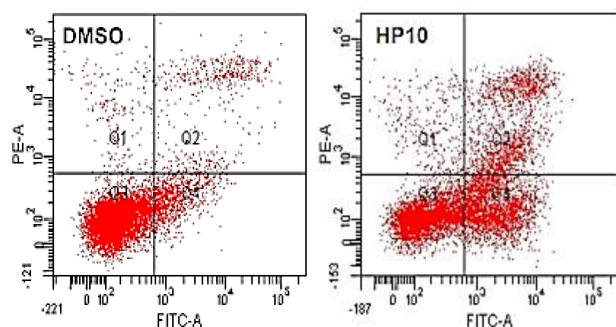

Fig. 5. Flow cytometric analysis by Annexin V and 7-AAD staining of HeLa cells treated with HP10 or solvent control (DMSO) for 24 hours.

It is important to mention that even though other extracts did not cause formation of apoptosis related 89 kDa cleaved PARP-1, the level of full length PARP-1 protein found to be diminished, indicating the presence of an alternative mechanism.

Since our data suggested that HP10 extract at its IC_{50} value induced apoptosis, we further reveal the mechanism in the cytotoxicity of HP10 using flow cytometric analysis. In apoptotic cells, the membrane phosphatidylserin is translocated from the inner to outer layer of the plasma membrane and Annexin V has ability for binding cells with exposed phosphatidylserin (Lee *et al*., 2013). With fluorochrome APC conjugation to Annexin V, it is possibly to determine even early stages of apoptosis (Wlodkowic *et al*., 2009, 2011). We have used APC Annexin V and 7-Amino-Actinomycin, which is a vital dye in our flow cytometry analysis and found that HP10 increased apoptotic cell numbers at its IC_{50} values in HeLa cells (Fig. 5).

When the anticancer effect of *H. perforatum* was searched in the literature, it is seen that there are more studies related to its photosensitizing effect. In addition, there are studies showing that extracts have antiproliferative and apoptotic effects on various cancer types. In these studies, the effects on eritroleukemic cell lines and breast cancer cells were more investigated and their results showed that *H. perforatum* has promising effects on cancer cells.

In our study the anticancer effects of *H. perforatum* samples collected from different habitats were investigated on some types of cancer cells and positive results were obtained in accordance with previous studies (Roscetti *et al*., 2004; Skalkos *et al*., 2005; Mirmalek *et al*., 2016; Valletta *et al*., 2018). In our study, besides the anticancer effects of the plant, the effects of different habitats on activity were investigated and compared. Physical, chemical properties and genotypes of plants show variations depending on environmental factors, such as region, temperature, height, CO_2 ratio, UV-B ratio, proportional humidity, rainfall retention rate, evapotranspiration, stream regimes, agriculture and proximity to the settlement areas. These factors deeply effect of the physical, chemical and vegetative properties of plants, consequently cause changes in ingredient combinations and amounts. These changes also affect the biological activities of medicinal plants. In our study, the ethanol extracts of *H. perforatum* collected from eleven different locations were studied and they showed cytotoxicity with varied potencies the depending on cell type. Although all the tested extracts showed to induce autophagy along with increased level of LC3-II and decreased level of p62, they exhibited different patterns regarding to apoptosis. Only HP10 coded sample was found to be increase the expression level of cleaved-PARP-1 and cleaved-caspase7, which are commonly used as apoptosis markers. Although other tested extracts did not caused formation of 89 kDa cleaved PARP-1, they diminished the level of full length PARP-1 protein. Further studies are required to determine the mechanism of this PARP-1 downregulation. HP10, the sample that induce the levels of cleaved apoptosis markers, was collected from the non-agricultural area of Ilgaz Mountain National Park (Çankırı Ilgaz) collected from the highest altitude. The region is located in the Öksin part of the Euro-Siberian Floristic Region and it is the transition zone between Central Anatolia and Western Black Sea Regions (Gümüs *et al*., 2002). Our results show that the anticancer effect of *H. perforatum* species is promising and may vary depending on height and environmental conditions.

Conclusions

According to our results we suggest that the ethanol extracts of *H. perforatum* has antiproliferative effects on human cervical cancer cell line (HeLa), human breast cancer cell line (HCC-1937), human osteosarcoma cell line (U2OS) probably by using autophagy and apoptosis processes. Furthermore, our results show that different climates has effect on plant contents and therapeutic effects, thereby the amount of compounds that have anticancer activity in the plant seem to change with the altitude.

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