Nonapoptotic cell death induced by *Hypericum* species on cancer cells

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**ABSTRACT**

**Objectives.** There are approximately 400 *Hypericum* species that grow naturally in different geographic origins of the world. Those species have been used in different folk medicines and screened for their biological activity including cancer. Our country is an important place for *Hypericum* species which are known as “kantaron, binbirdelik otu, kan otu, kılıç otu, yaraotu, kuzukıran”. We therefore evaluated the possible cytotoxic/apoptotic activities, total phenolic content and antioxidant capacity of the crude methanol extracts *Hypericum adenotrichum* Spach. and *Hypericum olympicum* L. which are still used in Turkish folk medicine.

**Methods.** The total phenolic content and antioxidant capacity were determined by Folin-Ciocalteu and ABTS methods. Anti-growth effects were screened in human hepatoma (Hep3B) and rat glioma (C6) cell lines by the MTT and ATP viability assays. The cell death mode (apoptosis/necrosis) was investigated by fluorescence imaging and the level of caspase-cleaved cytokeratin 18 (M30), active caspase-3 and cleaved PARP (poly (ADP-ribose) polymerase).

**Results.** The results indicate that the crude methanol extracts of *Hypericum olympicum* L. and *Hypericum adenotrichum* have both anti-growth/cytotoxic activities on these cells in a dose dependent manner.

**Conclusion.** These extracts clearly induced non-apoptotic cell death in human hepatoma (Hep3B) and rat glioma (C6) cell lines.

**Keywords:** *Hypericum olympicum, Hypericum adenotrichum*, cell death, necrosis

**Introduction**

Cancer is one of serious public health problem worldwide and second cause of death in developed countries. Despite current treatment regimens used clinically, survival rate is far from satisfactory [1]. Resistance to chemotherapy and other targeted therapies is a main problem for cancer research. In this context, there is an inevitable need to newly improved treatment options [2].

Medical plants have wide range usage worldwide and some of them are becoming an important part of clinical practice following screening for anticancer activity [3]. Hence, novel anticancer drugs from...
medicinal plants continue to play a crucial role in human health of the world’s population [4].

*Hypericum* (Guttiferae or Hypericaceae) is a large genus of herb with approximately 400 species that grows widely at temperate region of the world and has been used as traditional medicinal plant for hundreds of years. There are 89 *Hypericum* species in our flora of which 43 are endemic [5-7]. Anti-inflammatory, anti-viral, anti-microbial, anti-fungal, anti-depressant, anti-oxidant and cytotoxic activity of different species of *Hypericum* genus have been already identified [8-12]. Moreover, recent studies have reported their antitumor and apoptosis-inducing activity in addition to inhibition of tumor invasion and metastasis [13-18]. The present study was focused on the cytotoxic/apoptotic effect of crude methanol extracts from *Hypericum olympicum* and *Hypericum adenotrichum* (endemic) against on human hepatoma (Hep3B) and rat glioma (C6) cell lines. In addition, we investigated that the total phenolic content and antioxidant capacity of *Hypericum adenotrichum* and *Hypericum olympicum* extracts. This study reports the first time that both *Hypericum olympicum* and *Hypericum adenotrichum* have cytotoxic activity on these cells and trigger cell death in a non-apoptotic manner.

**Methods**

**Plant Materials**

Collection, authentication and extraction of *Hypericum olympicum* and *Hypericum adenotrichum* were described previously [19]. The crude extracts of *Hypericum adenotrichum* and *Hypericum olympicum* were dissolved in DMSO as a stock solution (100 mg/ml) and stored at -20ºC. The content of *Hypericum adenotrichum* extract (HAE) and *Hypericum olympicum* extract (HOE) were determined by GCXGC-TOF/MS method in York University, England. The content of *Hypericum olympicum* (unpublished data) and *Hypericum adenotrichum* has been published by Sarimahmut et al. [20].

**Determination of Total Phenolic Content and Antioxidant Capacity**

The HAE and HOE were prepared with mixed of methanol/water (50:50, v/v%) for determination of total phenolic content and antioxidant capacity. The Folin-Ciocalteu assay was used for the quantification of total phenolic content of HAE and HOE [21]. Absorbance at 750 nm was read using a Varian Cary 50, Australia spectrophotometer after incubating the reaction mixtures (Lowry A: 2% aqueous Na₂CO₃ in 0.1 M NaOH; Lowry B: 0.5% CuSO₄ aqueous solution in 1% NaKC₄H₄O₆ solution; Lowry C: prepared freshly as mixture 50 ml Lowry A and 1 ml Lowry B). Folin-Ciocalteu reagent was diluted with H₂O at a volume ratio 1:3 prior to use. The extracts (0.1 ml), 1.9 ml of H₂O and 2.5 ml of Lowry C solution were mixed and the mixture was let to stand for 10 min. At the end of this period, 0.25 ml of Folin reagent was added at room temperature for 30 min for stabilization of the blue color. The assay was done in duplicate. Results were expressed as mg of gallic acid equivalent (GAE) per g of lyophilized extract.

The total antioxidant capacity HAE and HOE were determined with ABTS method, as described by Sahin et al. [22]. The working solution was prepared by mixing two stock solutions of 20 mM ABTS and 2.45 mM potassium persulfate solution and allowed to react for 12–16 h at room temperature in the dark before use. The procedure for HAE and HOE were performed by adding 0.10 ml extract, 3.90 ml of ethanol and 1 ml of the ABTS+ radical cation solution, which was diluted with ethanol at a ratio of 1:10, and the absorbance at 734 nm was recorded against blank after 6 min. The results were expressed as milligram Trolox equivalent (TE) per gram of lyophilized extract.

**Cell Culture and Treatments**

Human hepatoma (Hep3B) and rat glioma (C6) cell lines were cultured in RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37ºC in a humidified atmosphere containing 5 %CO₂. Further dilutions of extracts dissolved in DMSO were made in culture medium to obtain the various concentrations ranging from 1.56 to 100 µg/ml. The highest concentration of DMSO did not exceed 0.1% (v/v) in the cell culture and the untreated/control cells received vehicle only (0.1% DMSO).

**The MTT Viability Assay**

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used to determine drug sensitivity of cells based on the linearly correlation between the number of viable cells and mitochondrial activity [23]. Hep3B and C6 cells were seeded in a 96-well plate at a density of 1x10⁴ cells/per well and subsequently treated with HAE and
HOE at the range of 1.56-100 µg/ml for 72 h. Each experiment was conducted twice in triplicates. At the end of the treatment period, 1:10 volume of MTT solution (5 mg/ml) was added to each well and allowed to formation of soluble formazan crystals by the living cells at 37ºC for an additional 4 h. 100 µl of solubilizing buffer (10% sodium dodecyl sulfate dissolved in 0.01 N HCl) was added to dissolve all crystals. After overnight incubation, the absorbance (Abs) was read by a spectrophotometer (FLASH Scan S12, Analytik Jena, Germany) at 570 nm and cell viability was determined by using the following formula: Cell viability (%) = \(\frac{100 \times (\text{Sample Abs})}{(\text{Control Abs})}\).

**ATP Viability Assay**

The ATP assay was used to determine the level of cellular ATP as an indirect marker in the assessment of cell viability and to verify the MTT results because of its sensitivity [24]. The seeding of cells, treatment conditions and the calculation of viability were all performed same as the MTT assay (see above). At the end of the treatment period, the ATP content of metabolically active cells was determined through the extraction of ATP from the cells following addition the luciferin-luciferase solution for luminometric measurement by using the ATP Bioluminescent Somatic Cell Assay Kit (Sigma, St. Louis, MO, USA) according to the manufacturer’s recommendations. The luminescence signal was measured at luminometer (Bio-Tek, USA).

**Determination of Cell Death Mode**

**Caspase-Cleaved Cytokeratin 18 (M30) Detection**

The level of the apoptosis-specific epithelial cell-derived caspase-cleaved cytokeratin 18 (ccK18, M30) was determined by using M30 Cytodeath ELISA kit (Peviva, Bromma, Sweden) according to the manufacturer’s instructions. Hep3B cells were seeded at a density of 1x10^4 per well of 96-well plate and treated with 100 µg/ml of HAE and HOE for 72 h to obtain maximum cell death. C6 rat glioma cell line was excluded in this assay because M30 assay detects only human-origin antigens. Paclitaxel (3.12 µM) was used as a positive control for apoptosis.

**Measurement of Active Caspase-3 and Cleaved PARP Levels**

The levels of the active caspase-3 and cleaved PARP (poly(ADP-ribose) polymerase) associated with the apoptotic process were determined using human caspase-3 (active) ELISA kit (Invitrogen Corporation, Camarillo, CA) and PARP Cleaved [214/215] ELISA kit (Invitrogen, CA) according to the protocols described in the manufacturers’ instructions. 1x10^6 Hep3B cells were seeded in 25 cm² flasks and subsequently treated with 100 µg/ml of the HAE and HOE for 72 h. Each experiment carried out in twice and results were given as fold change.

**Fluorescence Imaging of Nucleus and Annexin V-FITC Staining**

Phosphatidylserine translocation to the outside of the plasma membrane as an early apoptotic event was analyzed using the Annexin-V-Fluos kit (Roche, Mannheim, Germany). Propidium iodide (PI, 1 µg/ml) was used for the assessment of cells with damaged membranes due to primary necrosis or late apoptosis (secondary necrosis). Hoechst dye 33342 (5 µg/ml) was also added to the staining solution to evaluate apoptosis based on the nuclear morphology as described previously [25]. Hep3B and C6 cells were seeded at a density of 1x10^4 cells per well of 96-well plate and treated with HAE and HOE (50 and 100 µg/ml) for 48 h. Cells were visualized under fluorescence microscope after staining and considered as following:

- **Viable cells**: Annexin-V (-) and PI (-) as well as without nuclear pyknosis and/or chromatin condensation.
- **Early apoptotic cells**: Annexin-V (+) and PI (-) as well as with nuclear pyknosis and/or chromatin condensation.
- **Late apoptotic cells**: Annexin-V (+) and PI (+) as well as without nuclear pyknosis and/or chromatin condensation.
- **Non-apoptotic cells**: Annexin-V (-) and PI (+) as well as without nuclear pyknosis and/or chromatin condensation.

**Statistical Analysis**

All statistical analyses were performed using the SPSS 20.0 statistical software package for Windows. Significance was calculated using one-way analysis of variance (ANOVA). A value of \(p<0.05\) was considered statistically significant. The results are expressed as the mean ±SD (Standard deviation).

**Results**

**Total Phenolic Content and Antioxidant Capacity of**
Hypericum olympicum and Hypericum adenotrichum

Extracts

Total phenolic content of HAE and HOE were determined spectrometrically according to the Folin-Ciocalteu method and calculated as gallic acid equivalents (GAE). Total phenolic content was found 235±12 mg GAE/g for HAE and 333±13 mg GAE/g for HOE. Total phenol content was also determined in comparison with standard tannic acid. The potential antioxidant capacity of the extract was determined as milligrams TE/g lyophilized extract by ABTS method. Antioxidant capacity was found 303±5 mg TE/g for HAE and 725±2 mg TE/g for HOE. We showed that the total phenolic content and antioxidant capacity of HOE was considerably higher in HOE than HAE.

Anti-Growth Activities of Hypericum olympicum and Hypericum adenotrichum Extracts

Anti-growth activities of HAE and HOE against Hep3B and C6 cells were displayed in Figure 1,2 and

![Figure 1](image1.png)

Figure 1. The anti-growth effects after the treatment with varying doses of Hypericum adenotrichum extract and Hypericum olympicum extract for 72 h against Hep3B (a) and C6 (b) cancer cell lines by the MTT assay. *Denotes statistically significant differences in comparison with control (p<0.05).

![Figure 2](image2.png)

Figure 2. The anti-growth effects after the treatment with varying doses of Hypericum adenotrichum extract and Hypericum olympicum extract for 72 h against Hep3B (a) and C6 (b) cancer cell lines by the ATP assay. *Denotes statistically significant differences in comparison with control (p<0.05).
Table 1. The IC₅₀ and IC₉₀ values of the *Hypericum adenotrichum* extract (HAE) and *Hypericum olympicum* extract (HOE)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Extract</th>
<th>MTT Assay <em>IC₅₀ (µg/ml)</em></th>
<th>MTT Assay <strong>IC₉₀ (µg/ml)</strong></th>
<th>ATP Assay <em>IC₅₀ (µg/ml)</em></th>
<th>ATP Assay <strong>IC₉₀ (µg/ml)</strong></th>
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<tr>
<td>Hep3B</td>
<td>HAE</td>
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<td>&gt;100</td>
<td>42.60</td>
<td>85.28</td>
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<tr>
<td></td>
<td>HOE</td>
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<td>35.62</td>
<td>61.84</td>
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<tr>
<td>C6</td>
<td>HAE</td>
<td>78.38</td>
<td>&gt;100</td>
<td>43.46</td>
<td>84.44</td>
</tr>
<tr>
<td></td>
<td>HOE</td>
<td>40.17</td>
<td>&gt;100</td>
<td>41.02</td>
<td>68.23</td>
</tr>
</tbody>
</table>

*IC₅₀ is defined as the dose inhibiting 50% of viability, **IC₉₀ is defined as the dose inhibiting 90% of viability

Table 1. Cancer cells treated with increasing doses of HAE and HOE (1.56-100 µg/ml) for 72 h resulted in explicit decrease of cell viability in a dose-dependent manner. According to MTT assay results, the cell viability is significantly reduced at 25, 50 and 100 µg/ml doses (p<0.05) (Figure 1). On the other hand, results obtained from ATP assay revealed that cell viability is reduced more significantly at 50 and 100 µg/ml doses (p<0.05) (Figure 2) in Hep3B and C6 cell lines. HOE exhibited much better cytotoxic effect with IC₅₀ value of 26.30 µg/ml whereas HAE with IC₅₀ value of 42.04 µg/ml in Hep3B cells. However, the higher IC₅₀ values were determined in C6 cells after treatments with HOE (40.17 µg/ml) and HAE (78.38 µg/ml) (Table 1).

Based on morphological evaluation by phase contrast microscopy, prominent cytotoxic activity was observed in both Hep3B and C6 cells with HAE and HOE (100 µg/ml, 72 h), respectively (Figure 3). These examinations were found in accordance with the viability results.

**Non-apoptotic Cell Death Induced by Hypericum adenotrichum and Hypericum olympicum Extracts**

Following determination of potential anti-growth activity of HAE and HOE (100 µg/ml) in cancer cells, we further investigated the mode of cell death at first by fluorescence imaging based on nuclear morphology. In this context, cells were incubated with Hoechst dye 33342, which stains both alive and dead cells after treatment with HAE and HOE (50 and 100 µg/ml) for 48 h. We observed that the nucleus of dying cells does not have pyknosis and/or chromatin condensation, which are well-known features of apoptosis. Subsequent evaluation of phosphatidylserine (PS) translocation to the outside of membrane as a hallmark of early apoptosis was performed via Annexin V-FITC staining in addition to

**Figure 3. Images of Phase Contrast Microscopy.** Cells were treated with 100 µg/ml *Hypericum adenotrichum* extract (HAE) and *Hypericum olympicum* extract (HOE) for 72 h. Controls included untreated cells. A scale bar represents 10 µm.
PI staining in the assessment of plasma-membrane integrity. We observed prominently increment in the PI stained cells compared only Annexin-V-FITC stained cells (early apoptotic cells) as well as positivity for both of them (Annexin +/PI+) indicating late apoptotic and/or necrotic cells (Figures 4 and 5). When considering the lack of pyknosis and/or chromatin condensation, we suspected that the cells were actually dying by necrosis. Activation of caspases and presence of the caspase cleaved cytokeratin 18 (ccK18, M30) and/or cleaved poly (ADP-ribose) polymerase (PARP) are also hallmarks of apoptosis. As shown in Figure 6, we found that neither the caspase activation nor the cleavage of CK18 and PARP occur after treatment with HAE and HOE (100 µg/ml), implying that these cells do not undergo apoptosis with this treatment (Figure 6).

**Discussion**

Cancer is a serious ailment that has been estimated as the second leading cause of death in humans [26].
For this reason, there has been an extensive research on various biological sources for an effective treatment. In this regard, natural products as a source of effective anti-cancer agents have attracted considerable attention from scientists. Today, a substantial number of anticancer agents used in the clinic are derived from natural sources including plant [27, 28]. Since the plants are used in traditional medicine worldwide and several have been screened for their anticancer properties, we evaluated the possible cytotoxic activities of the *Hypericum adenotrichum* (endemic) and *Hypericum olympicum*, which are still used in Turkish folk medicine. In addition, we investigated that the total phenolic content and antioxidant capacity of *Hypericum adenotrichum* and *Hypericum olympicum* extracts. We found that the total phenolic content and antioxidant capacity were considerably higher in HOE than HAE. To our knowledge, there is no data the phenolic content and antioxidant capacity of HAE and HOE in literature. Therefore, we performed a detailed literature review of the *Hypericum* genus. Alipour et al. [29] showed that the total phenolic content of extract of *Hypericum fursei* was exhibited 274 ± 9.6 mg GAE/g. The total phenolic content of water extracts of *Hypericum undulatum* was found to be

![Figure 5. Fluorescence images of nuclei stained with Hoechst dye 33342 (left column, blue), Annexin-V-FITC (middle column, green) and propidium iodide (right column, red) after treatment with 50 and 100 μg/ml *Hypericum adenotrichum* and *Hypericum olympicum* for 48 h in C6 cells. Yellow arrows show the presence of Annexin-V-FITC staining.](image-url)
ranged from 119.90 to 191.77 mg GAE/g TE [30]. Zorzetto et al. [31] determined the antioxidant capacity of Hypericum species by ABTS method and found 251.2±29, 218.3±9.4, 323.1±49.9 and 766.5±43.3 µmol/g TE for Hypericum reflexum Ifonche, Hypericum reflexum La Esperanza, Hypericum canariense and Hypericum grandifolium respectively.

There are several studies on the anticancer activity of various Hypericum species against different type of cancer. For example, in a study, the protective effects of water extracts of Hypericum perforatum, Hypericum androsaemum and Hypericum undulatum were shown in colon cancer cells [32]. Hypericum sampsonii extract was found anti-growth and apoptotic effects on lung, liver and stomach cancer cells [33]. Hypericum perforatum extract have showed significantly inhibited prostate tumor growth [34]. Recently, Li et al. [14] examined that Hypericum ascyron extract has cytotoxic activity against different cancer cell lines. In our previous study, we demonstrated that the extract of Hypericum adenotrichum and Hypericum olympicum have cytotoxic activity on lung cancer cells by inducing apoptosis [19]. In this study, we showed that HAE and HOE exhibited significant anti-growth activity against human hepatoma (Hep3B) and rat glioma (C6) cell lines in a dose dependent manner. HOE was found more effective to cancer cells than HAE as the IC50 value (Table 1). The cytotoxic effects of HAE and HOE may be due to composition of plants. It is known that Hypericum adenotrichum and Hypericum olympicum contains compounds such as hypericin, pseudohypericin, hyperforin [35, 36]. Hypericin and hyperforin have a potent cytotoxic effect through inducing apoptosis in cancer cells [37, 38].

In our study, we investigated the mode of cell death resulted from HAE and HOE (50 and 100 µg/ml) for 48 h by fluorescence imaging. Late apoptotic/necrotic cells (positivity for PI staining, and negativity for Annexin-V-FITC staining) were observed both in HAE and HOE treatments. Also, it is showed that the nucleus of cells did not have pyknosis and/or chromatin condensation, any increment of PARP and M30 level in Hep3B and C6 cancer cells after treatment. These results demonstrated that the cells dying by necrosis after the treatment with HAE and HOE. In our previously studies, we found that HAE and HOE have cytotoxic effects by inducing apoptosis in human breast and lung cancer cells [19, 20]. In another study, Ozmen et al. [39] reported that petroleum ether extract of Hypericum adenotrichum induced apoptosis through on caspase 3 activation and PARP cleavage in HL-60 cells. It has implied that HAE and HOE may induce different cell death modes in different cell lines. The possible reasons for this outcome may be related to the genetic and phenotypic differences between the cell lines.

**Conclusions**

In conclusion, Hypericum adenotrichum and
Hypericum olympicum have potential anti-growth activity against human hepatoma (Hep3B) and rat glioma (C6) cell lines and they induce cell death via necrosis. Therefore, the potential cytotoxic activity of these plants needs further in vitro and in vivo experiments to elucidate the mechanism of action.

Conflict of interest
The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing
We appreciate the Uludag University for project numbered BUAP(F)-2014/3, providing us with the kits and chemicals.

References