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# Cytotoxic Activity and Apoptosis Induction of *Hypericum scabrum* L.

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**Background:** One of the acquired biological hallmarks of tumor multistep development is the resistance of cancer cells to apoptosis; therefore, induction of apoptosis is an important therapeutic approach. *Hypericum* species are spread throughout the world and have been investigated for their biological properties.

**Objectives:** Our previous studies had demonstrated cytotoxicity of *Hypericum scabrum* L. methanol extract against some tumor cell lines, suggesting the species for further studies. The objectives of the present study were to determine the most cytotoxic fraction of *Hypericum scabrum* L. and to assess the apoptosis induction ability of the most effective fraction as well as its methanol extract. The laboratory evidence has been presented to support the potency of Iranian Traditional Medicine (ITM) medicinal plants as a source of different biological activity surveys and drug discoveries.

**Materials and Methods:** The present research is a descriptive study. The sampling strategy was based on ITM data of cancer phytotherapy. *Hypericum scabrum* was collected from Alborz province, Iran (2012) and the herbarium specimen was taxonomically identified. The petroleum ether, dichloromethane, and methanol fractions have been evaluated for cytotoxicity against M-CF7, A-549, HT-29, and HepG-2 cell lines through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT assay. The apoptosis induction ability has been assessed by activated caspase-3 inspection and Annexin V FITC/PI (propidium iodide) assays.

**Results:** Di-chloromethane fraction demonstrated $IC_{50}$ values of 25.72 μg/mL and 24.73 μg/mL against HT-29 and HepG-2 cell lines, respectively and $IC_{50}$ values of petroleum ether fraction were 22.6 μg/mL and 18.31 μg/mL against HT-29 and HepG-2, respectively. The methanol fraction did not show cytotoxic activity. Both the methanol extract and the petroleum ether fraction of *Hypericum scabrum* L. revealed apoptosis induction ability.

**Conclusions:** Considering the strong historical background about the therapeutic potential of the genus *Hypericum* and the considerable cytotoxic and apoptosis induction ability of *Hypericum scabrum*, this species is suggested for future biological studies.

*Keywords:* Traditional Medicine; Caspase-3; Apoptosis; Annexin V Protein

## 1. Background

Cancer affects a lot of people all over the world (1) and in many cases cancer cells resist chemotherapy (2). One of the acquired biological hallmarks during tumor multistep development is the development of cancer cells resistance against programmed cell death, known as apoptosis (3). The essential role of apoptosis during tumor progression and chemotherapy resistance have been well defined (4) and restoring apoptosis is an important therapeutic approach (5). In other words, the apoptosis induction is important as the main mechanism of the cytotoxic effect of the cancer chemotherapeutic agents (6).

There are many common and distinctive natural products known as chemotherapeutic agents against cancer cells (7). The plants kingdom plays a especial role in such performance by introducing the taxanes, vinblastine, and some other drugs (8). There are still so many hopes to find novel lead compounds from natural sources (9).

The genus *Hypericum* contains over 400 species, which have been spread throughout the world, except Antarctica (10). Traditional Chinese medicine have introduced, at least, thirty medicinal plants of this genus, which are currently in use (11). In Europe, this genus has been used since ancient times. Dioscorides had introduced different kinds of this plant for its wound healing, diuretic, and emmenagogue properties (12). Also, Pliny had noted emmenagogue properties of these plants (13). The plants of this genus were also in use during medieval Iranian Traditional Medicine (ITM) for different therapies such as wound and old sores healing (14). They are still in use in present Iranian (15, 16), Indian (17), Turkish (18, 19), and Italian (20) traditional medicine for different healing properties. Nowadays, the most famous member of this genus is *Hypericum perforatum* (21).

This plant has been investigated for its several different biological activities e.g. antidepressant, antiviral, antimicrobial, and anti-inflammatory properties (22). One of the most reported healing properties of this genus even from ancient time (12) to the present is its wound healing char-

acteristic (17, 23). There are a number of studies that have been focused on the evaluation of biological activities of the genus *Hypericum*. *Hypericum perforatum* has modulated apoptosis in vivo, in neutrophils of patients with Behcet's disease (24), also its hyperforin has inhibited the growth of various tumor cell lines from rat and human origin and exhibited a dose dependent generation apoptosis against tumor cells, in vitro (25). Besides *Hypericum perforatum,* the other members of this genus have been investigated and evaluated for some biological activities e.g. antimicrobial properties of *Hypericum elongatum* (26), *Hypericum scabrum,* and *Hypericum capitatum* (27); anti-inflammatory properties of *Hypericum gentianoides* (28); anticonvulsant properties of *Hypericum scabrum* (29); and cytotoxic activity and apoptosis induction of *Hypericum sampsonii* (30). Different species of this genus have been introduced as medicinal plants in the Traditional Chinese Medicine (e.g. *Hypericum ancherii*, *Hypericum bellum*, *Hypericum geminiflorum*, *Hypericum wightianum*, *Hypericum perforatum*). They have been used in various therapeutic areas and consequently have been studied for evaluation of several biological activities (11). The above mentioned reputations suggest strong potency of these species to be surveyed for new drug discoveries.

## 2. Objectives

The methanol extract of *Hypericum scabrum* has demonstrated cytotoxic activity in our previous studies (31). In the present study, cytotoxic activity of 3 different fractions of *Hypericum scabrum* L. has been investigated by MTT assay against some tumor cell lines (MCF-7, A-549, HT-29, and HepG-2). The apoptotic induction ability of the methanol extract and the most cytotoxic fraction has also been evaluated against MCF-7 cell line. The laboratory evidence presented in this study, supports the potency of ITM medicinal plants as a source for different biological activity surveys and drug research.

## 3. Materials and Methods

The present research is a descriptive study. The statistical analysis, including 1-way ANOVA and Tukey posttest was carried out with Graphpad InStat software.

### 3.1. Plant Material

*Hypericum scabrum* was collected from Alborz Province, Iran (2012) and authenticated by botanists at the Traditional Medicine and Materia Medica research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran. A voucher specimen was deposited at the TMRC herbarium for future reference (3499-TMRC). This plant was shade dried and then ground.

### 3.2. Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), (Gibco, Auckland, New Zealand), RPMI 1640 medium, Penicillin-Streptomycin, MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide), Phosphate-buffered saline (PBS) (Sigma, St. Louis, MO, USA), and DMSO (Dimethyl Sulfoxide) (Merck, Hohenbrunn, Germany), were used to evaluate the cytotoxic activity. Moreover, petroleum ether, dichloromethane, and methanol (Merck, Hohenbrunn, Germany) were used for plant fractionation. The NucView™ 488 Caspase-3 assay kit for live cells (Biotium, Hayward, CA, USA) and Annexin V-FITC Apoptosis Detection kit (BioVision Research Products, Mountain View, CA, USA) were used in apoptosis assays. Also we used 5-Flourouracil (5-FU) (Sigma, St. Louis, MO, USA) as the positive control.

### 3.3. Cell Lines

MCF-7 (human breast adenocarcinoma), HepG-2 (hepatocellular carcinoma), A-549 (non-small cell lung carcinoma), and HT-29 (colorectal adenocarcinoma) cells were obtained from Pasteur Institute, Tehran, Iran. MCF-7 and HT-29 cell lines were cultured in DMEM with 5% FBS for MCF-7 cells and 10% FBS for HT-29 cells, while the other two cell lines were cultured in RPMI 1640 medium with 10% FBS to maintain the desired growth. All cell lines were treated with 1% penicillin-streptomycin and were kept in a humidified incubator at 37°C in an atmosphere of 5% $CO_2$.

### 3.4. Extraction

Ten grams of the dried powdered *Hypericum scabrum* was macerated with methanol at room temperature for 24 hours (thrice). The methanol extract was further concentrated using a rotary evaporator at 40°C and the dried extract was kept in 2°C - 8°C for future assays.

### 3.5. Fractionation

Dried powdered *Hypericum scabrum* (20 g) was macerated with petroleum ether, dichloromethane, and methanol successively at room temperature for 24 hours thrice. The fractions were concentrated and kept in the same condition as the extract.

### 3.6. Preparation for the Assays

The samples were dissolved in DMSO: 10 mg/mL to make the stock solutions of each sample. Serial dilutions were prepared accordingly from the above stock solution to reach the final concentrations (DMSO 1%).

### 3.7. MTT Assay

Cell viability was assessed through MTT assay, as mentioned earlier (32, 33) with different concentrations of the extract/fractions. The cells were seeded in 96-well plates at $6 \times 10^3$ for MCF-7, $5 \times 10^3$ for HT-29, $15 \times 10^3$ for HepG-2, and $8.5 \times 10^3$ for A-549. After 24 hours, the former medium was replaced with fresh medium containing different concentrations of the extracts to be tested, i.e. 100,

50, 25, 12.5, 6.25, and 3.125 μg/mL. After 72 hours exposure of the cells to each sample at 37°C, the medium was replaced with fresh medium containing MTT, with a final concentration of 0.5 mg/mL. The cells were incubated for another 4 hours, then the medium containing MTT was removed, and the remaining formazan crystals were dissolved in DMSO. The absorbance was recorded at 570 nm with an enzyme-linked immunosorbent assay (ELISA) reader (TECAN), using 5-FU as the positive control.

The relative cell viability (%) related to control wells was calculated by [A] samples/[A] control × 100, where [A] samples was the absorbance of the test sample and [A] control was the absorbance of wells containing cells, cell culture medium, and DMSO 1%. The viability versus Log concentration curves were graphed by the Microsoft Excel program and the $IC_{50}$ values were assessed.

### 3.8. Assessment of Apoptosis Induction

There are different methods for studying apoptotic cells (34). In the present study, we have utilized Caspase-3 assay in live cells and Annexin-V/PI staining methods to investigate apoptotic induction ability of the methanol extract and the most cytotoxic fraction of *Hypericum scabrum* against MCF-7 cell line.

### 3.9. Caspase-3 Assay in Live Cells

Caspases are normally presented in their inactive form (zymogens) in the cytosol and different pathways turn them into active forms. The significant member of this family in the execution phase of apoptosis is caspase-3, which can activate other caspases (35). The apoptosis ability of the samples has been evaluated by observing active caspase-3 in the test medium (36). MCF-7 cells were cultured with the same above-mentioned method. After 24 hours of incubation, MCF-7 cells were treated with *Hypericum scabrum* methanol extract and petroleum ether fraction in the concentrations of 40 and 8 μg/mL, respectively. After 20 hours of incubation, the medium was replaced with PBS and 10 μL/mL from NucView™ 488 Caspase-3 assay kit for live cCells was added. After 20 minutes of incubation in the darkness, the results were evaluated with an inverted florescent microscope (HUND) and the images recorded by a digital camera (Canon 600D). The green florescence documented the presence of active caspase-3 at the cytosol and subsequently the active apoptotic cascade. The excitation and emission wavelengths were 450 and 490 nm, respectively.

### 3.10. Annexin-V/Propidium Iodide Staining Method

To qualify the apoptotic cell death, the Annexin V-FITC Apoptosis Detection kit (37) from BioVision Research was used. MCF-7 cells were cultured and after 24 hours, they were treated with *Hypericum scabrum* methanol extract and the petroleum ether fraction in the concentrations of 40 and 8 μg/mL, respectively. After 20 hours incubation, the medium was replaced with the kit binding buffer (100 μL). Then, 2.5 μL/mL Annexin V-FITC and Propidium Iodide were added consecutively. After 5 minutes incubation in the dark and at room temperature, the results were examined utilizing an inverted florescent microscope (HUND, Germany) and recorded by a digital camera (Canon 600D, Japan). The excitation and emission wavelengths were 450 and 490 nm, respectively. 5-FU was used as the positive control for both apoptosis induction assays.

### 3.11. Necrosis Induction

To induce necrosis, the cells were treated with chloroform (10 μL/mL), just before the apoptosis assays. Necrosis and apoptosis are the main mechanisms of the cell death. The first is considered as accidental and uncontrolled cell death and the other is an scheduled physiologic cell death (38). In the present study, chloroform was used to induce necrosis (39) in order to distinguish apoptosis from necrosis.

## 4. Results

### 4.1. MTT Assay

The methanol extract of *Hypericum scabrum* has demonstrated cytotoxic activity in our previous study (31). In the present study, cytotoxic activity of 3 different fractions of *Hypericum scabrum* L. was investigated by MTT assay and the results are presented in the Table 1.

**Table 1.** Results of Cytotoxicity Assay (Aerial Parts of *Hypericum scabrum*) Against MCF-7, A-549, HT-29, and HepG-2 Cell Lines

| Extracts | $IC_{50}$, μg/mL | | | |
|---|---|---|---|---|
| | MCF-7 | A-549 | HT-29 | HepG-2 |
| **Methanol extract** | a | a | b | b |
| **Methanol fraction** | b | b | b | b |
| **Di-chloromethane fraction** | 43.89 | 31.34 | 25.72 | 24.73 |
| **Petroleum ether fraction** | 11.87 | 17.31 | 22.6 | 18.31 |
| **5-FU (positive control)** | 1.53 | 3.91 | 4.75 | 7.57 |
| **P value** [c] | 0.0016 | 0.0385 | 0.2490 | 0.3453 |

[a] $IC_{50}$ values were reported from our former study (31).
[b] $IC_{50}$ values greater than 100 μg/mL were considered as inactive.
[c] The considered groups were the $IC_{50}$ results of each column (A cell line and the $IC_{50}$ values obtained by Di-chloromethane and Petroleum ether fractions). Each reported $IC_{50}$ result came from average of 3 tests.

### 4.2. Statistical Analysis

Comparing the results with 1-way ANOVA and unpaired t test (using the GSTAT software) revealed that $IC_{50}$ values for MCF-7 and A-549 caused by dichloromethane and petroleum ether were significantly different (P = 0.0016, P = 0.0385) and for HT-29 and HepG-2 cells, were not significant. So we decided to perform the rest of study (apoptosis induction evaluation) by petroleum ether fraction against MCF-7 cell line regarding lower $IC_{50}$ values and partially notable difference between test groups.

### 4.3. Caspase-3 Assay in Live MCF-7 Cells

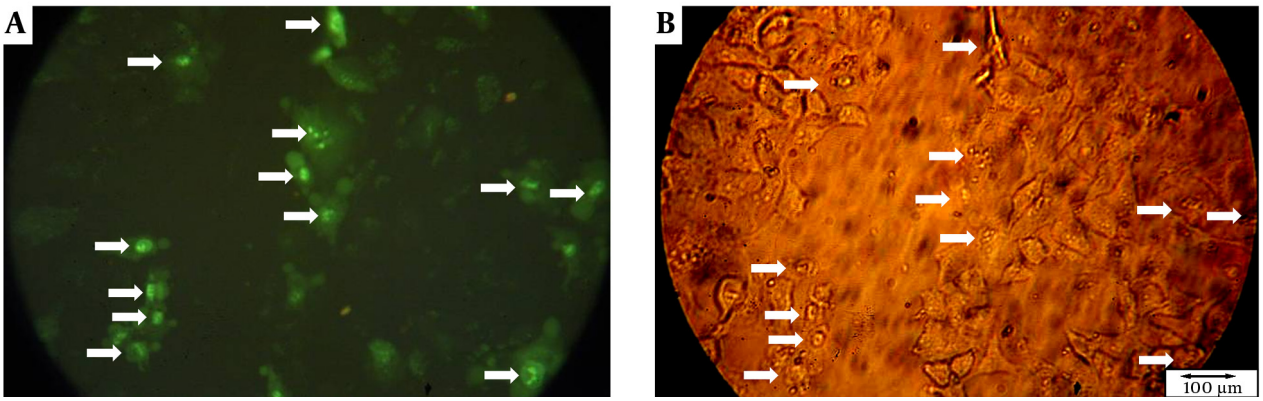
The following figures would exhibit the possible apoptosis induction ability of the *Hypericum scabrum* methanol extract and petroleum ether fraction by observing activated caspase-3 in live cells (Figures 1 - 3).

### 4.4. Annexin-V/Propidium Iodide Staining Method in Live MCF-7 Cells

The following figures would also exhibit possible apoptosis induction ability of the *Hypericum scabrum* methanol extract and petroleum ether fraction by observing dual and unicolor fluorescent in live cells (Figures 4 - 7).

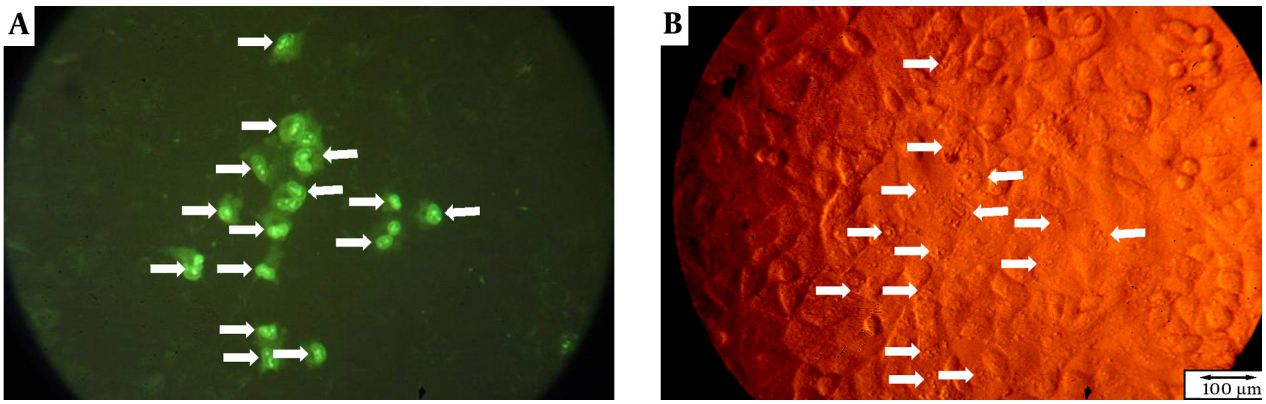
The brilliant green florescent from condensed nuclei in caspase-3 assay and morphologically changed MCF-7 cells, which shows condensed nuclei and shrunk cells' cytoplasm from both methanol extract and petroleum ether fraction confirmed apoptosis induction (Figures 1 and 2). The positive control, 5-FU, also confirmed the tests accuracy (Figure 3). The Annexin-V/PI staining method, which could be considered as a differentiator between apoptotic and necrotic cell death also exhibited both apoptotic and necrotic dying cells in the field. At a glance, these results are controversial i.e. the presence of both necrotic and apoptotic suffering cells in the same field (Figures 4 and 5). But the tests on positive controls, 5-FU and chloroform, qualified the apoptotic cell death. This statement could be estimated visually. Besides, the morphology of the cells in the light field could be helpful. According to the differentiating nature of the present

**Figure 1.** Results of the Caspase-3 Assay on the Live Cells of MCF-7 Cells Treated With 40 µg/mL of Methanol Extract of *H. scabrum* L



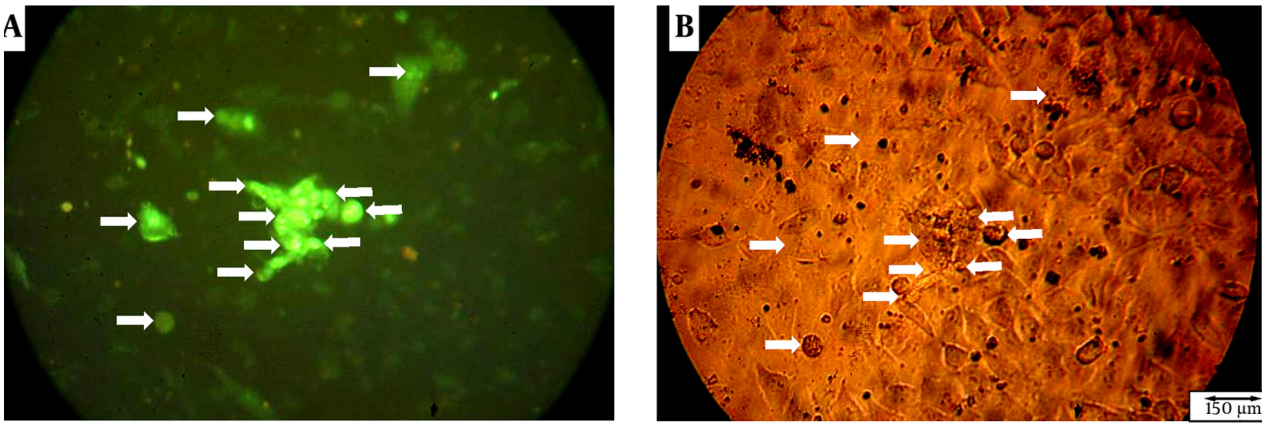

'A' remarks fluorescent and 'B' remarks the light microscope view of the same field.

**Figure 2.** Results of the Caspase 3 Assay on the Live Cells of MCF-7 Cells Treated With 8 µg/mL of Petroleum Ether Fraction of *H. scabrum* L



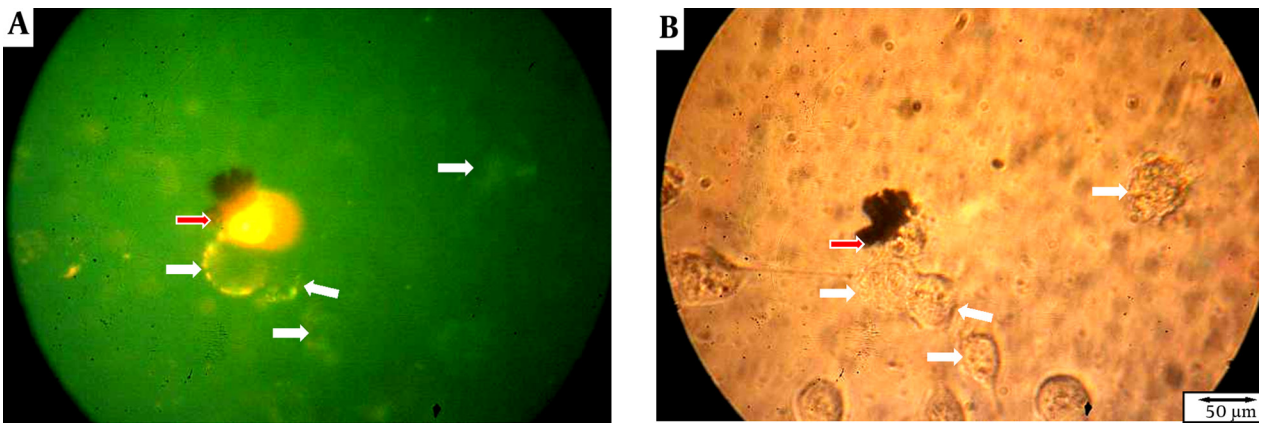

'A' remarks fluorescent and 'B' remarks the light microscope of the same field.

**Figure 3.** Results of the Caspase-3 Assay on the Live Cells of MCF-7 Cells Treated With 0.5 µg/mL of 5-FU



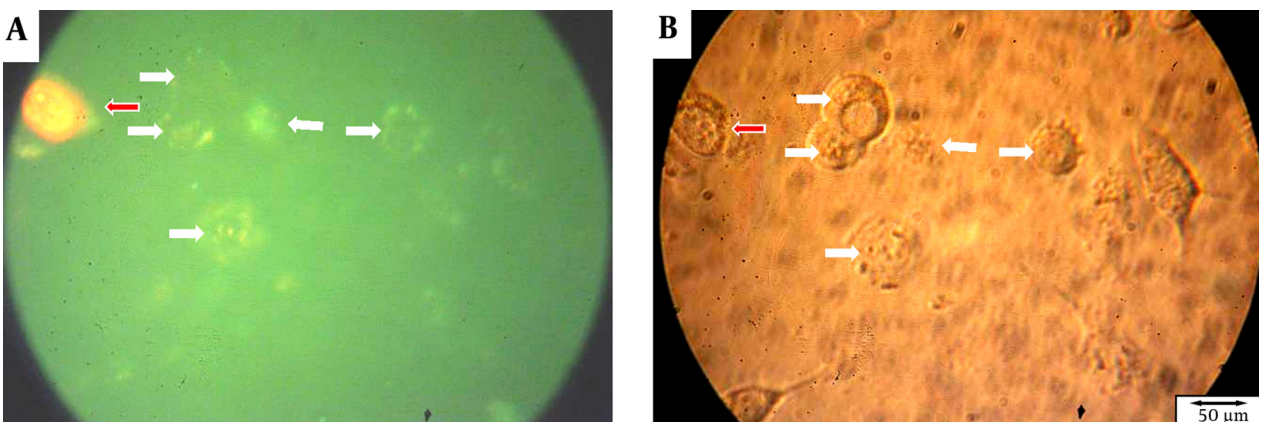

'A' remarks fluorescent and 'B' remarks the light microscope of the same field.

**Figure 4.** Results of the Annexin V-FITC/PI Assay on the Live Cells of MCF-7 Cells Treated With 40 µL/mL of *H. scabrum* L Methanol extract



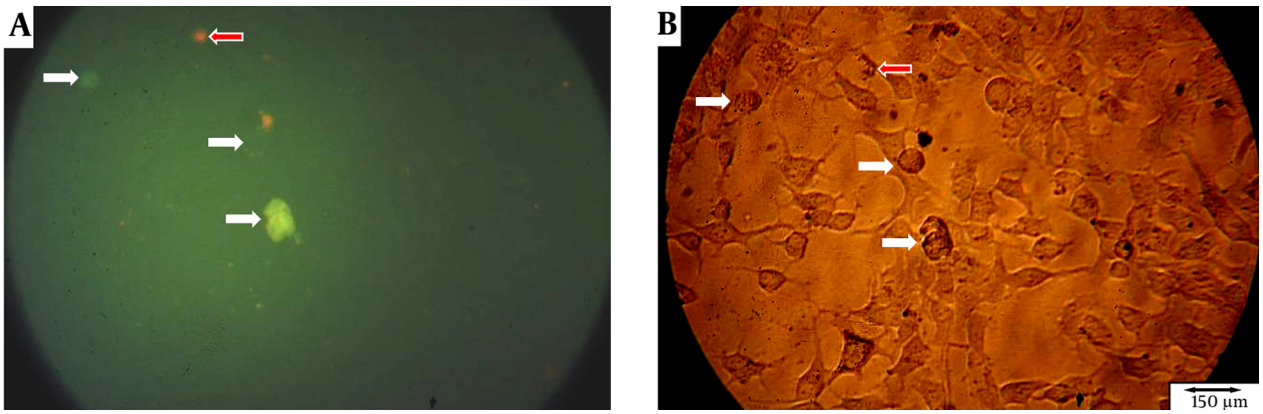

'A' remarks fluorescent and 'B' remarks the light microscope of the same field.

**Figure 5.** Results of the Annexin V-FITC/PI Assay on the Live Cells of MCF-7 Cells Treated With 8 µL/mL of *H. scabrum* L. Petroleum Ether Extract



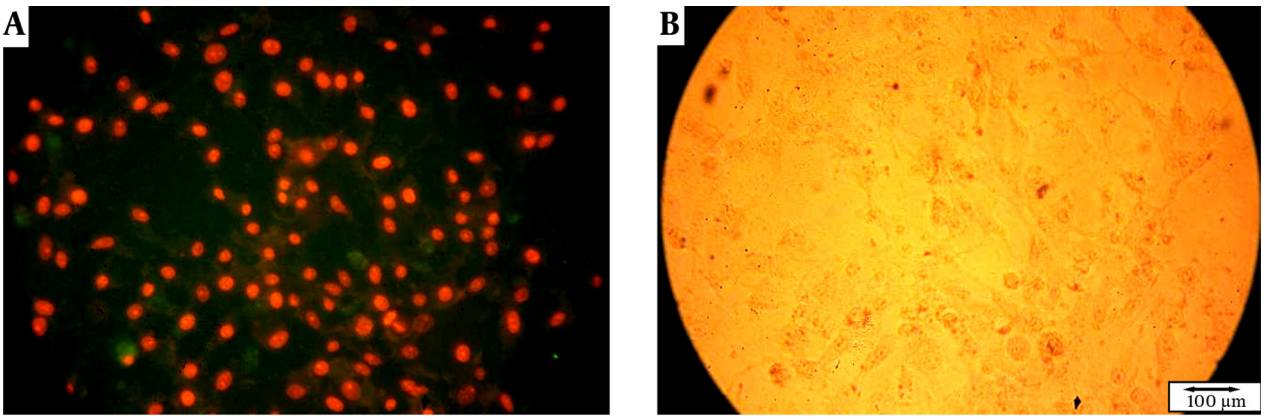

'A' remarks fluorescent and 'B' remarks the light microscope of the same field.

**Figure 6.** Results of the Annexin V-FITC/PI Assay on the Live Cells of MCF-7 Cells Treated With 0.5 µg/mL of 5-FU




'A' remarks fluorescent and 'B' remarks the light microscope of the same field.

**Figure 7.** Results of the Annexin V-FITC/PI Assay on the Live Cells of MCF-7 Cells Treated With 10 µL/mL of Chloroform




'A' remarks fluorescent and 'B' remarks the light microscope of the same field.

test between apoptosis and necrosis, it could be claimed that the cytotoxic activity of *Hypericum scabrum* L. could be due to apoptotic induction.

## 5. Discussion

The genus *Hypericum* comprises medicinal plants, which have been used from ancient times and throughout the old world. The plants of this genus have been investigated for different biological activities and there are also strong statements about their wound healing characteristic and medicinal properties in ITM. Therefore, it was selected for evaluation in our study (14). Laboratory results revealed that its methanol extract exhibited cytotoxic properties only against MCF-7 and A-549 cells, however, the two fractions showed cytotoxicity against all 4 examined cell lines. This could be due to the increase in cytotoxic agent(s) concentration by fractionation. The significant P value for the $IC_{50}$ amounts made us to choose petroleum ether fraction as the appropriate subject for apoptosis induction assay. The apoptotic potential of the methanol extract and the most appropriate fraction were examined on MCF-7 cells.

Evaluating the activity of the activated caspase-3 is a proper indicator of apoptosis induction. The activated cytosolic caspase-3 cleaves the substrate and releases the specific nucleus dye, which could stain the DNA. Along with the fluorescent shining nuclei of apoptotic cells, the morphologic changes of nucleus during apoptosis such as chromatin condensation and nucleus separation could be observed by fluorescent microscopy. These changes were demonstrated in the reported figures. Figures 1 and 2 demonstrated the brilliant fluorescence caused by activated caspase-3 in the condensed nuclei of MCF-7 cells and the light microscopic field view certified the apoptotic process by obvious shrunk cytoplasm. The positive control (Figure 3) confirms the apoptosis induction ability of the methanol extract and the petroleum ether fraction. Also, fluorescent field (Figures 6 and 7) showed both dual and unicolor cells, which confirmed necrotic and apoptotic cell death, respectively. The mor-

phological changes in MCF-7 cells of the light field view also certified apoptosis. The presence of more apoptotic cells regarding the necrotic ones, confirmed the apoptosis induction ability of methanol extract and petroleum ether fraction. Figures 4 and 5 approved the accuracy of the tests.

Other studies have been carried out about *Hypericum* genus and its apoptosis induction ability, and most of these studies have been focused on *Hypericum perforatum* and its active molecule hypericin (40). These studies have reported both apoptosis induction ability (41) or antiapoptotic effect of *Hypericum perforatum* (42) and this effect has suggested to be related to flavonoides (43). The results of the present study have once again showed that different medicinal plants of the genus *Hypericum* could be good sources of biologically active ingredients. The MTT and the apoptosis induction ability (Caspase-3 and Annexin V-FITC assay) results indicate the apoptotic potential of *Hypericum scabrum* L. and introduce it as a proper candidate for future cancer studies. Besides, the capacity of ITM to introduce new source of plants for different biological activity surveys and drug research has also been well considered. As it was mentioned above, we have used ITM information about cancer remediation to select *Hypericum perforatum* for the present evaluation and though the study has introduced a new route of plant selection for cancer research, more and detailed studies of molecular mechanisms of apoptosis induction are necessary.

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## Authors' Contributions

Maryam Hamzeloo-Moghadam designed the study, also she was the supervisor and advisor of the study and helped with the manuscript preparation. Amir Khalaj collected the plant material and performed all executional phases of the study and helped with the manuscript preparation. Maryam Malekmohammadi collected the plant material and identified its scientific name.

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