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INDUCEMENT OF APOPTOSIS IN HEPG2 CELLS BY TOTAL EXTRACT OF *Scutellaria barbata* AND *Pseuderanthemum bracteatum* Imlay

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ABSTRACT

This study aimed to evaluate the effect of total extract of Scutellaria barbata and Pseuderanthemum bracteatum Imlay on HepG2 cell line. The results showed that total extract reduced HepG2 cell density for 48 hours of treatment. The total extract induced the changes expression of apoptosis-related genes in HepG2 cells including Bcl-2 and caspase family. Moreover, the ratio of apoptotic cells was associated with an increase of total extract concentration.

Keywords: apoptosis, gene expression, *Pseuderanthemum bracteatum* Imlay, *Scutellaria barbata*, total extract.

1. Introduction

Scutellaria barbata (*S. barbata*) is a species of flowering plant native to Asia in the mint family, *Lamiaceae*. It is known as *Ban Zhi Lian* in Chinese and its English common name is barbed skullcap. *S. barbata* is an herb used a lot in traditional Chinese medicine. Some of its well-known uses are the ability to repel toxic from the body, induction of apoptosis in prostate cancer, and treatment of metastatic breast cancer. It helps in treating patients with tuberculosis and appendicitis. The plant is good for people with hepatitis and cirrhotic ascites. Moreover, *S. barbata* is also included in herbal remedies for inflammation and traumatic injuries (Dai et al., 2013). The plant extract from *S. barbata* D. Don. has also been proved to have antiproliferative effect on cultured human uterine leiomyoma cells, hepatoma cell line R-HepG2, human colon cancer cells, and human lung cancer cell line A549 (Goh et al., 2005; Kim et al., 2008; Tang et al., 2012; Yin et al., 2004). Even though it is widely known and used in many different diseases, the main focus of research nowadays is the plant's active ability in fighting cancer.

Pseuderanthemum bracteatum Imlay (*P. bracteatum* Imlay) in the *Acanthaceae* family is a common plant in Vietnam. The leaves of this plant are often used in the treatment of multiple trauma, bleeding, diarrhea, and stomach ulcers (Padee et al., 2010).

The plant roots also contain some compounds that have high biological activity such as lupeol, lupenone, betulin, pomolic acid, etc. These compounds, especially lupeol and betulin, was confirmed to have the active antimicrobial, antioxidant and cytotoxicity ability on HepG2 liver cancer cells and MCF7 breast cancer cells (Król et al., 2014). On the other hand, Bhanuz Dechayont and his team has also pointed out that the phenolic compounds found in *Pseuderanthemum* have high antioxidant activities, and the dry leaf extract macerated with 95% EtOH showed cytotoxic activity against COR-L23 and A549 (Dechayont et al., 2015), thus showing the potential use of total *P. bracteatum* Imlay extracts in the treatment of cancer.

Most of the studies have been performed previously using only single extracts of plants to treat cancer. However, the effectiveness of treatment can be increased considerably, if possible, by combining total extracts of many plants that have the same anti-cancer effect. Therefore, this study aimed to use the combination of total plant extracts from *S. barbata* and *P. bracteatum* Imlay to induce apoptosis on HepG2 cells.

2. Materials and methods

2.1. HepG2 cell culture

HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin/streptomycin) at 37^{o}C in a 5% CO_2 atmosphere (all purchased from Gibco). The cells were exposed to fresh culture medium every two days.

2.2. Cell density measurement

HepG2 cells were cultured in 35mm dishes and treated in triplicate with different concentrations of total extract of *S. barbata* and *P. bracteatum* Imlay (1, 10, 100 μg/ml) in 48h with a control dish. The total extracts were prepared by the combination of *S. barbata* extract and *P. bracteatum* Imlay extract with ratio 1:1. The cells were detached by trypsin-EDTA 0.25%, then the cell number was determined by adding Trypan Blue (1:1) and counting the number of living cells using the haemocytometer red blood cell counting chamber. Analysis was based on the number of living cells and dead cells counted. Cell density was assessed by the formula: (total number of cells – the number of dead cells) / total cell count.

2.3. RNA extraction

Following treatment with the specific reagents, the HepG2 cells were harvested and washed twice with sterile phosphate-buffered saline (PBS). Their total RNA was extracted using a Ribospin™ Total RNA Purification Kit, according to the manufacturer's instructions (GeneAll Biotechnology, Seoul, Korea). The quality and quantity of the RNA sample were assessed using the spectrophotometer (NanoVue Plus Spectrophotometer, GE Healthcare Life Sciences). RNA samples with a ratio of A_{260}/A_{280} between 1.8 and 2.0 were aliquoted and stored in liquid nitrogen until used.

2.4. Quantitative real time-PCR analysis

RNA samples stored away were used for the real time PCR reaction. 1 µl of total RNA from each sample was used together with 2 µl of primers as described below (Shalini et al., 2015), 10 µl Mix Ro-Lox, 1 µl RTAse, in a total volume of 20 µl in each reaction. The PCR reaction was performed in triplicate by one cycle of 45^{o}C for 15 min; one cycle of 95^{o}C for 2 min; 40 cycles of 95^{o}C for 10 sec, annealing temperature used according to the specific set of primers for 15 sec and 62^{o}C for 15 sec; and 71 cycles of 60^{o}C for 30 sec. To compare the level of each RNA transcript to the control GAPDH, the Livak method was used.

Table 1. *Primer sequence used in quantitative real time-PCR reaction*

Gene	GenBank accession number	Sequence	Product size (bp)
GAPDH	NM 002046	F: 5'-GAAGGTCGGAGTCAACGGATTT-3' R: 5'-CTGGAAGATGGTGATGGGATTTC-3'	223
p53	NM 000546	F: 5'-GAGCACTGCCCAACAACAC-3' R: 5'-ATGGCGGGAGGTAGACTGA-3'	251
Bcl-2	NM 000633	F: 5'-TTCTTTGAGTTCGGTGGGG-3' R: 5'-CAGGAGAAATCAAACAGAGGC-3'	194
Bax	NM 138764	F: 5'-CTTTTGCTTCAGGGTTTCATC-3' R: 5'-CACTCGCTCAGCTTCTTGGT-3'	113
Caspase 3	NM 032991	F: 5'-ATGGAAGCGAATCAATGGAC-3' R: 5'-ATCACGCATCAATTCCACAA-3'	242
Caspase 8	NM 033356	F: 5'-ATGCAGGGGCTTTGACCACGAC-3' R: 5'-TCCCCCTGACAAGCCTGAATAAAA-3'	292
Caspase 9	NM 032996	F: 5'-GCGAACTAACAGGCAAGCA-3' R: 5'-CCAAATCCTCCAGAACCAAT-3'	144

2.5. Flow cytometry

To analyze cell death after drug treatment, the HepG2 cells were harvested using trypsin – EDTA, washed twice with PBS 1X and centrifuged to remove supernatant. The cells were then incubated in 200 µl binding buffer containing Annexin-V-FITC (5 µl/ml) and PI (5 µl/ml) at room temperature in the dark for 30 minutes. After centrifugation and removal of supernatant, 200 µl PBS 1X was added to the tube containing cells before flow cytometric analysis on the BD Accuri^{TM} C6 Flow Cytometer 2.

2.6. Statistical analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was used to analyze the variance for the means of multiple groups. Statistical analysis was performed using Sigma Plot program (version 11.0), and significant differences were considered at values of $P \leq 0.05$. Results were expressed as mean ± standard error.

3. Results

3.1. Effect of combination of total plant extracts on HepG2 cell density

The group treated with extracts at concentration of 1 µg/ml has an density of 100.67 x 10^4 cell/dish, decreased nearly a half compared to the control group (191.67 x 10^4 cell/dish) (Figure 1). Cell density in the dish treated with extracts at concentrations of 10 µg/ml and 100 µg/ml also decreased by 62.33 x 10^4 and 58.00 x 10^4 cell/dish, respectively.

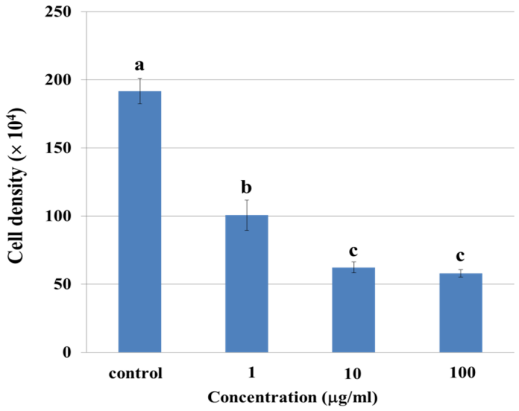

Figure 1. *Effect of combination of extraction at different concentrations on HepG2 cell density. a, b, c: significant difference with P ≤ 0.05*

3.2. Effect of combination of extracts at different concentrations on the expression of apoptosis-related genes on HepG2 cells

As depicted in Figure 2, treatment with *S. barbata* and *P. bracteatum Imlay* total extracts markedly increase the mRNA level of p53 in HepG2 cells compared with control dish. There was a down-regulation in mRNA expression levels of Bcl-2 in dishes treated at 1 µg/ml and 10 µg/ml, 100 µg/ml (Figure 2). However, for the mRNA expression of Bax, analyzing real time PCR results showed a high up-regulation (Figure 2).

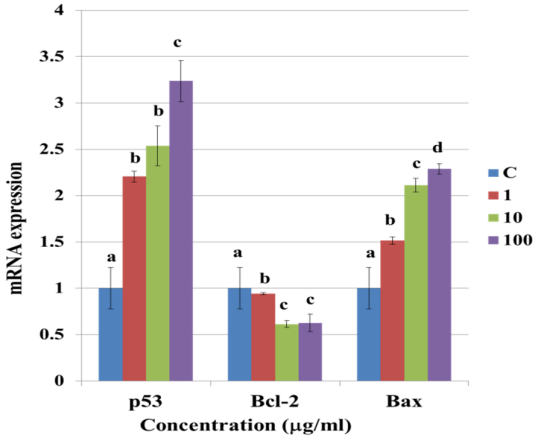

Figure 2. *Effect of the combination of total plant extracts at different concentrations on the expression of p53, Bcl-2, and Bax in cells. a, b, c: significant difference with P ≤ 0:05*

According to Figure 3, both Caspase 9 and Caspase 3 displayed an increase in mRNA expression level after being treated with total plant extraction at different concentrations for 48h. Moreover, the real time PCR results showed in Figure 3 depicted the up-regulation of Caspase 8 mRNA expression after being exposed to total *S. barbata* and *P. bracteatum Imlay* extraction at 1 µg/ml and 10 µg/ml for 48h. Nevertheless, at concentration 100 µg/ml, Caspase 8 experienced a sudden drop in mRNA expression.

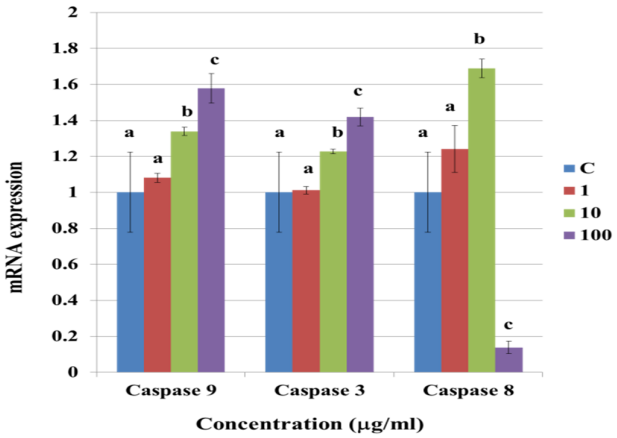

Figure 3. *Effect of the combination of total plant extracts at different concentrations on the Caspase family expression in cells. a, b, c: significant difference with $P \leq 0.05$*

3.3. Cell death stages in apoptosis

The percentages of early apoptotic, late apoptotic, necrotic and live cells are shown in Figure 7. Flow cytometry analysis revealed that the ratio of Annexin V – positive and PI – positive cells (the apoptotic cells) treated at 10 µg/ml was 2.1% which was higher than groups treated at 1 µg/ml (1.0%) and control dish (0.2%).

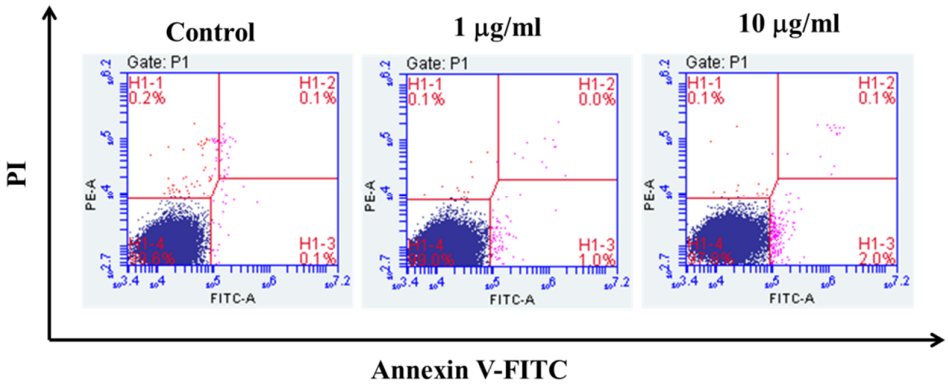

Figure 4. *Cell apoptosis determined by Annexin V-FITC staining. Flow cytometer analysis of the apoptotic and necrotic cells (H1-1: necrotic; H1-2: late apoptotic; H1-3: early apoptotic; H1-4: live) after 48 hours of incubation with 1 µg/ml and 10 µg/ml of total S. barbata and P. bracteatum Imlay extract, respectively*

4. Discussion

The Bax protein forms a heterodimer with Bcl-2 and can function as a trigger for apoptosis. Its expression is regulated by the tumor suppressor gene p53, when the amount of p53 in the cell increases, Bax will also be activated simultaneously. It will interact and strengthen the opening of voltage – dependent anion channel, VDAC (Shi et al., 2012), leading to the loss of cell membrane ability and the release of cytochrome c and other pro-apoptotic factors from the mitochondria, leading to activation of caspase (in this case, Caspase 9) (Weng et al., 2015). Through the extrinsic pathway, the caspase cascade is activated by surface Death Receptors. Death Receptor such as Fas binds the FasR receptor at the receptor's extracellular surface; this activates the death domains at the cytoplasmic tail of the receptor. Then, through a Death domain – Death domain interaction, adaptor protein FADD is recruited. FasR, FADD and pro-Caspase 8 form the Death-Inducing Signaling Complex (DISC) and activate Caspase 8. Once activated, the initiator Caspases will, in turn, activate the executioner caspases: Caspase 3, 6, and 7 (Fischer et al., 2008).

When treated with the combination of total plant extracts at different concentration of 1 μg/ml and 10 μg/ml, 100 μg/ml, the expression of p53 in the cells spiked up, Bax expression also increased, but Bcl-2 expression decreased. In the caspase cascade, expression of Caspase 9 and 3 kept going up as the level of concentration got higher. Caspase 8 expression at 1 μg/ml and 10 μg/ml was also increasing. Simultaneously, pictures taken under fluorescent microscope of cell nuclei stained with DAPI dye expressed DNA fragmentation in the cell nucleus treated with total plant extracts. This implies that after 48 hours of treatment with the combination of two plant extracts, HepG2 cells have begun to go into apoptosis. The flow cytometry analysis further confirmed this idea as it showed that when concentration of total plant extract increase, the ratio of apoptotic cells also increase (from 1.0% at 1 μg/ml to 2.1% at 10 μg/ml). The ratio of necrotic cells remained unchanged in both concentration may imply that this combination of total plant extract truly is effective on indulging cancer cells to go into apoptosis and not affect normal cells as long as it doesn’t reach the threshold.

However, there was a sudden drop in the expression of Caspase 8 at concentration 100 μg/ml. This unexpected decrease may imply that 100 μg/ml can be the threshold concentration for the Caspase 8 enzyme. Caspase 8 plays an important role in inducing Caspase 3 activation (Huang et al., 2011), but it can be seen clearly that Caspase 3 expression was still increasing at 100 μg/ml, despite Caspase 8 dramatic drop in expression, which proposed that at this concentration, Caspase 9 was the main factor that participates in the activation of Caspase 3.

5. Conclusion

The present study explored the effect of combination of *S. barbata* and *P. bracteatum Imlay* total extracts, which have been proved effective individually, on HepG2 cells.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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CẢM ỨNG SỰ CHẾT THEO CHƯƠNG TRÌNH Ở TẾ BÀO HEPG2 BẰNG CAO CHIẾT TỔNG TỪ CÂY BÁN CHI LIÊN (*Scutellaria barbata*) VÀ CÂY HOÀN NGỌC (*Pseuderanthemum bracteatum* Imlay)

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TÓM TẮT

Trong nghiên cứu này, chúng tôi đánh giá tác động của cao chiết tổng của cây bán chi liên và cây hoàn ngọc trên tế bào ung thư gan HepG2. Kết quả cho thấy cao chiết tổng làm giảm mật độ tế bào HepG2 sau 48 giờ xử lí. Cao chiết tổng còn cảm ứng sự thay đổi sự biểu hiện của các gene liên quan đến sự chết theo chương trình của tế bào HepG2 như họ Bcl-2 và caspase. Hơn nữa, tỉ lệ tế bào chết theo chương trình cũng tương ứng với sự gia tăng nồng độ của cao chiết.

Từ khóa: cao chiết tổng, *Pseuderanthemum bracteatum* Imlay, *Scutellaria barbata*, sự biểu hiện gene, sự chết theo chương trình.