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THE EFFECT OF POGOSTONE ON VIABILITY, MEMBRANE INTEGRITY, AND APOPTOSIS OF LIVER CANCER CELLS

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Abstract – *Objective:* *The incidence of liver cancer is still high in many countries, including Iran. Drug resistance and various side effects are the main obstacles to treating this cancer. Herbs, which are traditionally used, are now widely regarded as treatment options for cancer. Pogostone is a natural substance isolated from Indian mint (Pogostemon cablin) and has various medicinal activities. This study aimed to determine the effect of Pogostone on liver cancer cell line (viability, membrane integrity, and apoptosis).*

Materials and methods: *The liver cancer cell line was prepared from Pasteur Institute of Iran and treated with appropriate concentrations of Pogostone. Cytotoxicity was determined by MTT, trypan blue, and lactate dehydrogenase assay. Apoptosis induction was evaluated by diphenylamine assay, Annexin V-FITC staining and a Real-time PCR test. Data were analyzed by SPSS statistical software using Tukey's test one-way analysis of variance.*

Results: *After all three time periods, a significant decrease in viability was observed ($p < 0.05$) in a concentration- and time-dependent manner. The cytotoxicity of Pogostone to liver cancer cells was in a concentration- and time-dependent manner. Pogostone significantly induced apoptosis compared to control cells ($p<0.05$). Treatment of liver cancer cells with Pogostone significantly reduced Bcl-2 gene expression ($p<0.05$). On the other hand, expression of all three Bax, p53, and caspase 3 genes showed a significant increase after treatment ($p<0.05$).*

Conclusions: *Pogostone had a concentration- and time-dependent toxic effect on liver cancer cells. It induced apoptosis by increasing the Bax to Bcl-2 ratio.*

KEYWORDS: *Pogostone, Liver Cancer Cell, Viability, Membrane Integrity, Apoptosis*

INTRODUCTION

Cancer is the second leading cause of death worldwide behind cardiovascular disease[1]. In 2015, approximately 90.5 million people worldwide were diagnosed with cancer[2]. In 2019, the annual incidence of cancer increased to 23.6 million people and 10 million deaths worldwide, representing an increase of 26% and 21% in the last decade, respectively[3].

Liver cancer or hepatocellular carcinoma is a cancer that occurs in the liver. It can be primary (originates in the liver) or secondary (liver metastasis). The global liver cancer incidence and mor-

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tality have been increasing[4,5]. Primary liver cancer is the sixth most common cancer worldwide and the fourth leading cause of cancer death. In 2018, it occurred in 841,000 people and resulted in the deaths of 782,000 people worldwide. Higher rates of liver cancer occur where hepatitis B and C are prevalent, including in Asia and sub-Saharan Africa. Men are more likely than women to develop liver cancer. Most diagnoses are between the ages of 55 and 65[6,7]. Most of the patients are currently diagnosed at advanced stages of the disease and are not suitable candidates for surgical resection of the tumor. Systemic chemotherapy with cytotoxic agents (5-Fluoracil, doxorubicin, cisplatin, and oxaliplatin) and targeted therapy with the tyrosine kinase inhibitor sorafenib are the main approaches for these patients; however, chemotherapy resistance, adverse effects and high costs remains are the major obstacles[8]. Extensive studies have been carried out in the last few decades to enhance the efficacy of anticancer drugs by overcoming chemoresistance.

Over the past millions of years, a large number of natural products have been used to treat a variety of ailments, despite the lack of scientific confirmation of their effectiveness and safety[9]. Following the development of various therapeutic side effects and drug resistance, conventional drugs are ineffective for the complete cure of some diseases. Thus, efforts are underway to find new drugs to complement or replace conventional therapies. Some plants have anti-cancer agents whose derivatives have been proven to be used to treat or prevent cancer in humans. In the 1950s, scientists began a systematic study of natural products as a source of beneficial anti-cancer substances. It has recently been suggested that the use of natural products has been the most successful strategy in the discovery of new drugs[10,11].

Indian mint (Pogostemon cablin) is a species of flowering plant in the Lamiaceae family, commonly referred to as the mint or nettle family. It is native to the island region of Southeast Asia including Sri Lanka, Indonesia, the Malay Peninsula, New Guinea, and the Philippines, and is also found in many parts of northeastern India[12]. Pogostone is one of the main components of Indian mint and has various biological activities. Pogostone has been reported to have potent anti-fungal, anti-bacterial, anti-inflammatory, and pesticide activities. Studies of its pharmacokinetic properties showed that Pogostone is readily absorbed with high bioavailability after oral administration[13].

Surgery, chemotherapy, and radiation therapy are common methods of treating most cancers. Liver tumors usually do not respond to chemotherapy, and chemotherapy has many side effects. Therefore, efforts to discover new therapies are always ongoing. The present study aimed to investigate the effect of Pogostone on the liver cancer cell line.

MATERIALS AND METHODS

Cell culture and treatment

For this experimental *in vitro* study, HepG2 cell line was obtained from the Pasteur Institute (Tehran, Iran) and cultured in Roswell Park Memorial Institute-1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). The cell culture was maintained at 37°C with 5% CO_2 in a humidified atmosphere. Pogostone (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) (Gibco, Grand Island, NY, USA) to a concentration of 3 mg/ml. The cells were treated with 1, 2, 4, 8, 16, 32, 64, and 128 µg/ml of Pogostone for 24, 48, and 72 hr for trypan blue, MTT, and lactate dehydrogenase tests. They were treated with the half-maximal inhibitory concentration (IC50) concentration of Pogostone for 24 hr for apoptosis tests. The study was approved by the Ethical Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (Code: IR.KUMS.MED.REC.1400.032).

Cell viability tests

The cells were seeded in a 96-well plate and incubated overnight to adhere to the bed. They were treated with Pogostone. For trypan blue staining, after trypsinization, the cell suspension was mixed with trypan blue stain solution (0.4%) (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 3 min at room temperature. A drop of the trypan blue/cell mixture was applied to a hemacytometer. The unstained (viable) and stained (nonviable) cells were counted by a microscope. The percentage of cell viability was calculated by dividing the number of viable cells by the number of total cells×100.

For 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay 50 µl of MTT solution (5 mg / 1 ml) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated in the dark. After 4 hr, 100 µl of DMSO was added to each well and the plate was placed on a shaker at room temperature

for 20 min. Absorbance was read at a wavelength of 570 nm against a reference wavelength of 630 nm using a plate reader and the percentage of cell viability was calculated by dividing the absorbance of the treated group by the absorption of the control group×100.

The IC50 values were calculated using GraphPad Prism version 6 software (La Jolla, CA, USA).

Cytotoxicity assay

This study was performed using the lactate dehydrogenase activity kit (Abcam Inc., Cambridge, MA, USA). After treatment, the medium on the cells was removed and after centrifugation, 50 µl of supernatant of each sample was added to the wells of a 96-well plate, and lactate dehydrogenase activity was measured per the kit instructions and using the standard nicotinamide adenine dinucleotide curve.

Apoptosis tests

After treatment, the cells were trypsinized and counted after centrifugation. $10^5 \times 5$ cells were suspended in 500 µl of binding buffer (BioLegend, London, UK). 5 µl of annexin V-FITC (Abcam Inc., Cambridge, MA, USA) and 5 µl of Propidium Iodide (Sigma-Aldrich, St. Louis, MO, USA) were added. Samples were incubated in the dark at room temperature for 5 min. Finally, 10,000 cell events in each sample were analyzed by DML program.

The percentage of DNA fragmentation after treatment was determined by diphenylamine assay as described by Cohen and Duke and the absorbance of samples was measured at 600 nm using a spectrophotometer.

Gene expression analysis

Apoptosis-related gene expression levels were evaluated by Real-time PCR. After treatment, total RNA was extracted from cells by TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The quantity and quality of the RNAs were verified by nanodrop spectrophotometer and electrophoresis using 1% agarose gel, respectively. Complementary DNA was synthesized using a cDNA synthesis kit (Vivantis Technologies, Selangor DE, Malaysia) according to the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. Real-time PCR was performed using SYBR Premix Ex Taq Technology (TaKaRa Bio Inc., Otsu, Shiga, Japan) on the Applied Biosystems StepOne Real-time PCR System. Gene expression analyses was quantified according to the $2^{-\Delta Ct}$ method. All the primers were designed using GeneRunner software and checked in NCBI Primer Blast. The primer sequences were as follows:

— Bax Forward: 5′-CCTGTGCACCAAGGTGCCGGAACT-3′
— Reverse: 5′-CCACCCTGGTCTTGGATCCAGCCC-3′
— Bcl-2 Forward: 5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′
— Reverse: 5′-GGTGCCGGTTCAGGTACTCAGTCA-3′
— p53 Forward: 5′-TAACAGTTCCTGCATGGGCGGC-3′
— Reverse: 5′-AGGACAGGCACAAACACGCACC-3′
— Caspase 3 Forward: 5′-CAAACTTTTTCAGAGGGGATCG-3′
— Reverse: 5′-GCATACTGTTTCAGCATGGCAC-3′
— GAPDH Forward: 5′-TCCCTGAGCTGAACGGGAAG-3′
— Reverse: 5′-GGAGGAGTGGGTGTCGCTGT-3′

Statistical analysis

The tests were repeated at least three times independently. All data are expressed as means ± standard deviation (SD). Comparisons between groups were performed by Tukey's test one-way analysis of variance and differences were considered significant when $p<0.05$.

RESULTS

The effect of Pogostone on cell viability

The effects of different concentrations of Pogostone on cell viability after 24, 48, and 72 hr were measured by Trypan blue and MTT tests (Figure 1). After all three time periods, a significant decrease in viability was observed in all concentrations ($p<0.05$). This decrease was in a concentration- and time-dependent manner. The IC50 values calculated in trypan blue test were 38.38±4.15, 19.43±4.37, and 2.01±0.17 µg/ml, respectively, and in MTT test were 15.72±2.82, 13.89±2.2, and 2.36±1.22 µg/ml for 24, 48 and 72 hr.

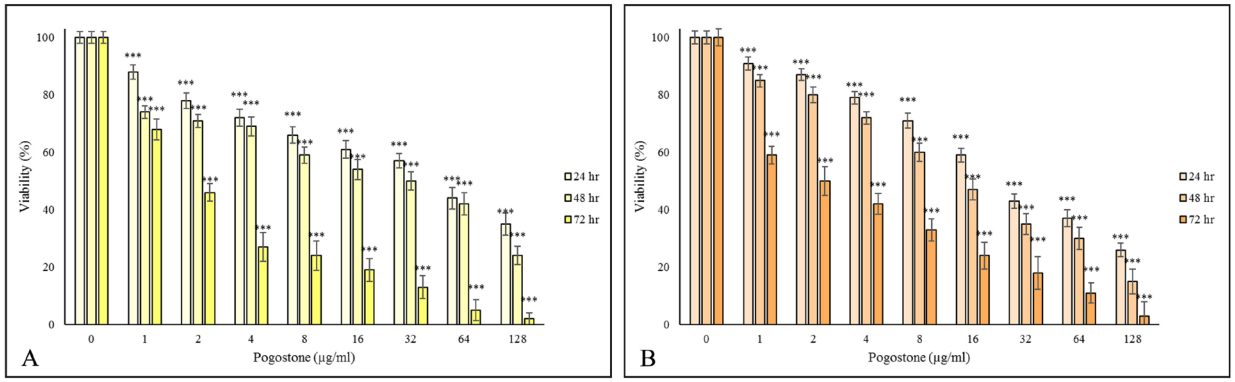

Fig. 1. Effect of Pogostone on the viability of liver cancer cells. Cell viability was assessed by (A) trypan blue staining and (B) MTT assay after 24, 48 and 72 hr. Control cells received the same volume of drug-free medium. *** Indicates $p<0.001$ compared to control.

The cytotoxic effect of Pogostone

As shown in Figure 2, the cytotoxicity of Pogostone to liver cancer cells was in a concentration- and time-dependent manner. This toxicity after 24 hr at concentrations of 8, 16, 32, 64 and 128 µg/ml, after 48 hr at concentrations of 4, 8, 16, 32, 64 and 128 µg/ml, and after 72 hr at concentrations of 2, 4, 8, 16, 32, 64 and 128 µg/ml, was significant ($p<0.05$).

The effect of Pogostone on cell apoptosis

Percentage of intact cells, early apoptosis, late apoptosis, and necrotic cells after treatment with IC50 concentration for 24 hr showed that there were significant differences between treated and control cells. The results showed that 98.2% of the control cells were live, 0.24% were early apoptotic cells, and 0.28% were late apoptotic cells. The treatment group showed a significant increase in early (28.6%) and late (3.28%) apoptotic cells (Figure 3).

The results of diphenylamine assay showed that Pogostone significantly induced apoptosis compared to control cells ($p<0.05$) (Figure 4).

The effect of Pogostone on the expression of Bax, Bcl-2, p53, and caspase 3 genes

Gene expression assays showed that treatment of liver cancer cells with IC50 concentration of Pogostone for 24 hr significantly reduced Bcl-2 gene expression ($p<0.05$). On the other hand, expression of all three Bax, p53, and caspase 3 genes showed a significant increase after treatment ($p<0.05$) (Figure 5).

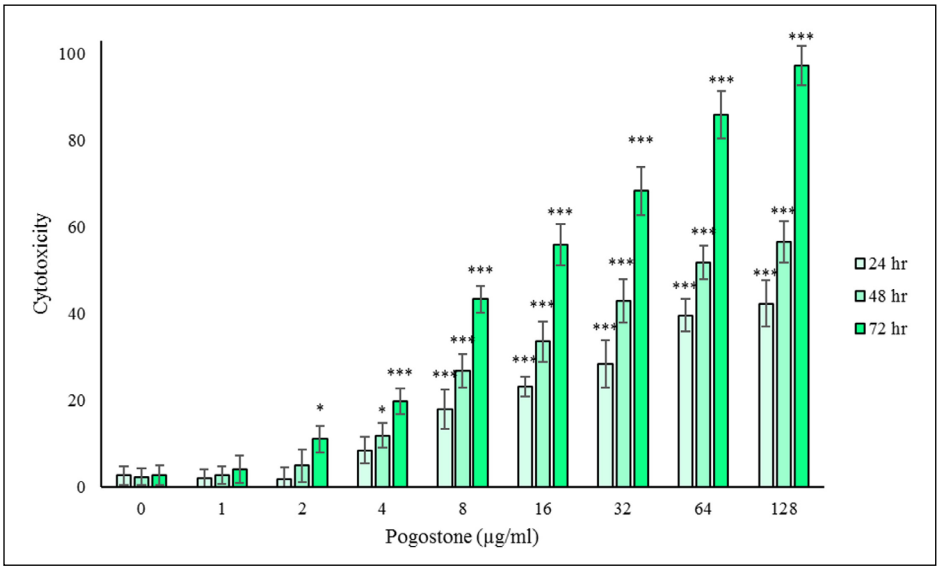

Fig. 2. Pogostone toxicity on liver cancer cells after 24, 48 and 72 hr was examined by lactate dehydrogenase activity assay. The control group cells received the same volume of drug-free medium. * Indicates $p<0.05$ and *** indicates $p<0.001$ compared to controls.

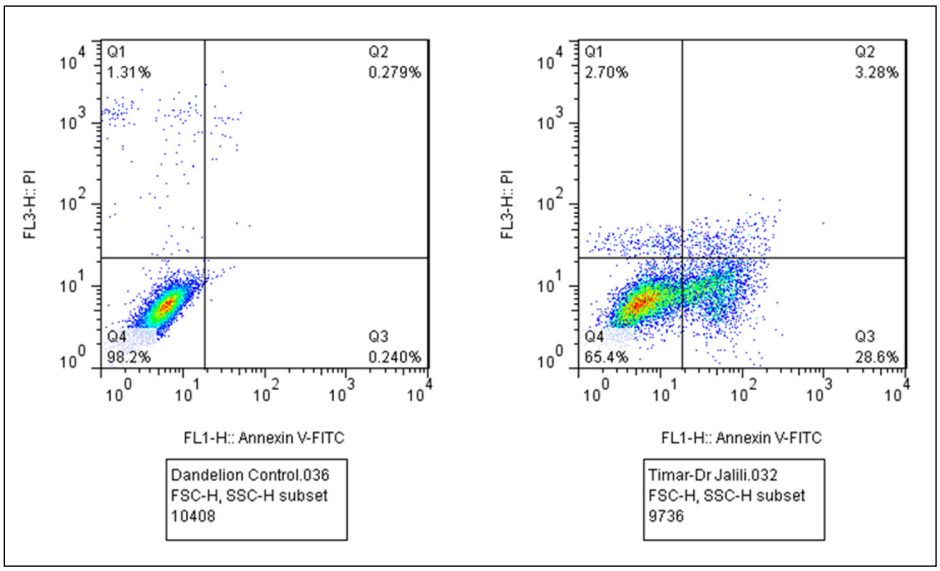

Fig. 3. The rate of apoptosis in liver cancer cells was measured by flow cytometry after 24 hr treatment with Pogostone. Control cells received serum-free culture medium.

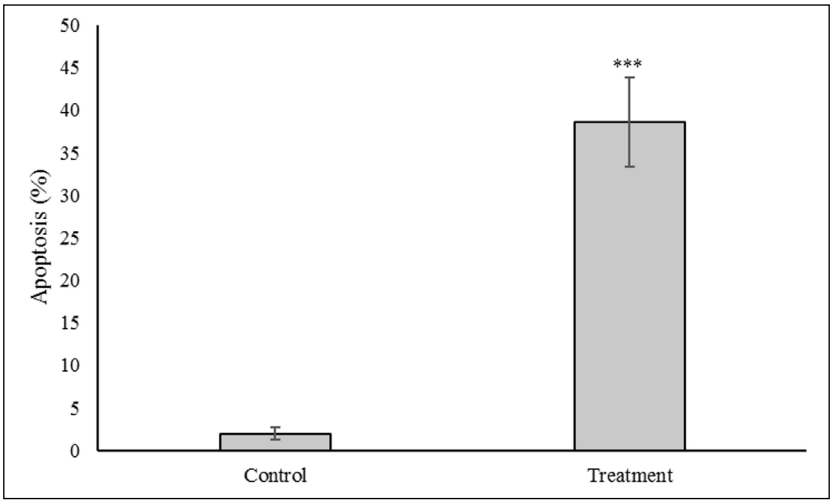

Fig. 4. Effect of Pogostone on the apoptosis of liver cancer cells. Cell apoptosis was assessed by Diphenylamine assay after 24 hr. Control cells received the same volume of drug-free medium. *** Indicates $p<0.001$ compared to control.

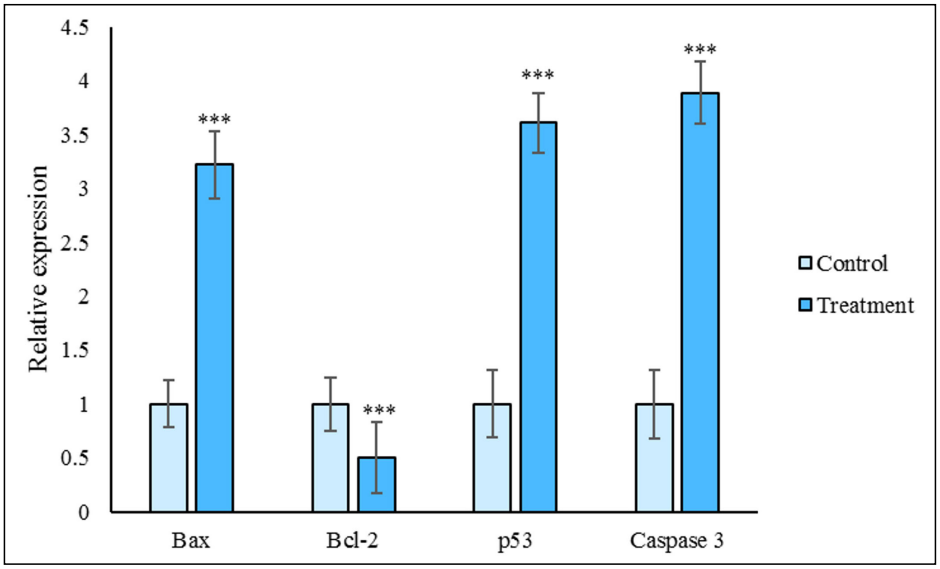

Fig. 5. The effect of Pogostone on the expression of apoptotic genes in liver cancer cells. Gene expression was assessed after 24 hr of treatment with IC50 concentration, and control group cells received the same volume of drug-free medium. *** Indicates $p<0.001$ compared to control.

DISCUSSION

In this project, the effect of Pogostone on the viability of liver cancer cells was first investigated. The results showed that after all three periods of treatment, the viability of the cells gradually decreased with increasing Pogostone concentration and time ($p<0.05$). Also, lactate dehydrogenase activity assay in cell culture medium after treatment and comparison with the control group showed that this decrease in viability was associated with cell membrane damage. In the later stages, cell death is accompanied by loss of membrane integrity and release of the cytoplasm contents out of the cell. Lactate dehydrogenase is a relatively stable enzyme that is measured to assess the presence of damage or toxicity to cells in cell culture media[14].

In a study, the anti-tumor activity of Pogostone on colorectal cells was evaluated *in vitro* using MTT assay. Pogostone showed significant anti-tumor activity against human colorectal cancer cell lines, particularly HCT116[15] which confirms our results. The level of IC50 obtained in this study was lower than in our study, which can be attributed to differences in the type and origin of the cell lines. Our data showed that Pogostone induced apoptosis in the liver cancer cell line. The ability to induce apoptosis and autophagy by this compound in the colorectal cell line was also investigated by Annexin V, tunnel test, transmission electron microscopy, and mRFP-GFP-LC3 fluorescence analysis. Results showed that Pogostone induced autophagy and apoptosis by regulating the expression of LC3-dependent, caspase-3, and caspase-7, and reduction of AKT / mTOR phosphorylation[15]. The induction of apoptosis in this study is consistent with our results.

Also *in vivo*, the anti-tumor effect of Pogostone was tested. 150 mg/kg Pogostone inhibited HCT116 tumor growth in immunocompromised mice with a 43.3% inhibition rate, reduced Ki67 expression, and induced apoptosis within three days[15].

The toxicity of Indian peppermint extract on some cancer cells has been confirmed in other studies. The plant suppressed cell growth in a dose-dependent manner in colorectal cancer cells HCT116 and SW480. In addition, it reduced cell growth in MCF7, BxPC3, PC3, and HUVEC cells. Exposure of HCT116 and SW480 cells activated p21 expression and suppressed the expression of cyclin D1 and cyclin-dependent kinase 4 (CDK4) in a dose-dependent manner. In addition, it decreased the expression of histone deacetylase 2 and the activity of c-myc and HDAC enzymes. The plant induced NF-κB transcriptional activity by increasing P65 nucleus translocation. These findings suggested that the plant exerted anti-cancer activity by reducing cell growth and increasing apoptosis in human colon cancer cells. Proposed mechanisms included inhibition of HDAC2 expression and HDAC enzyme activity and subsequent reduction of c-myc regulation and activation of the NF-κB pathway[16].

The compound, isolated from peppermint oil, also exerts anti-tumor activity against the ability of A549 human lung cancer cells, both *in vitro* and *in vivo*. Measurement of caspase activity showed that caspase-9 and caspase-3 activated mitochondrial-mediated apoptosis. Continuously, this substance inhibited xenograft tumors in the body. Further investigation of the underlying molecular mechanism showed that the MAPK and EGFR pathways may contribute to the anti-tumor effect[17].

Molecular studies showed that the mRNA expression of two apoptosis-promoting genes (p53, Bax, and caspase-3) is significantly increased by Pogostone. Also, the expression of Bcl-2 (anti-apoptotic gene) decreased after treatment.

Many proteins with anti-apoptotic and pre-apoptotic activity have been reported. The ratio between these proteins plays an important role in regulating cell death. The Bcl-2 family of proteins consists of anti-apoptotic and pro-apoptotic proteins that play an important role in the regulation of apoptosis. These proteins act mainly on the mitochondrial level. All members of the Bcl-2 family are located in the outer membrane of the mitochondria, where they are demyelinated and are responsible for membrane permeability by forming ion channels or by creating pores in the membrane. Bcl-2 is the first known protein in this family to be encoded by the BCL-2 gene on chromosome 18q21, which inhibits apoptosis without affecting cell proliferation. Bax protein is a member of the Bcl-2 family and promotes apoptosis. Bax / Bcl-2 ratio determines the occurrence of apoptosis in the cell. In many human cancers, Bcl-2 expression is increased, while Bax expression is decreased. This factor causes most cancer cells to become resistant to stimuli, including chemotherapy drugs[18].

The results of our studies showed that after 24 hr of treatment, decreased Bcl-2 expression, and increased Caspase 3 and Bax expression were observed. Bax/Bcl-2 mRNA ratio was increased in liver cancer cells after treatment. Therefore, the expression ratio of pre-apoptotic to anti-apoptotic proteins had changed to perform apoptosis.

CONCLUSIONS

Pogostone showed anti-cancer effects on liver cancer cells, which were associated with induction of apoptosis by increasing Bax/Bcl-2 expression.

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Conflict of Interests:

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Author contributions:

D. R. designed the experiments, supervised the research, and co-authored the manuscript. S. A, and H. N. performed the cell culture experiments. C. J. analysed data, wrote the manuscript and carried out one gene expression analysis of this work. All authors read and approved the final manuscript.

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