Combination effect of Daphne extract and Imatinib on Anti proliferation of the K562 cell line

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Research Article

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Abstract

Background

Modern and ancient medical sciences should be used to treat cancer. Herbal medicine is well known among the ancient medical sciences. Healing properties have been observed in some species of Daphne. The effect of Daphne plant extract on the k-562 cell line has been previously studied, and Gleevec is a well-known and effective drug in the treatment of chronic myelogenous leukemia.

Material and Methods

In this study, the simultaneous effect of using herbal medicine and a targeted therapy drug on the k-562 cell line was investigated. The presence of some species of Daphne in Iran motivated us to evaluate the cytotoxic activity of Daphne mucronata on human leukemia cancer cells. Anti-proliferative activity of the dichloromethane extract of daphne mucronate (Thymelaeaceae) as a new anticancer medicinal plant was evaluated. Apoptotic and necrotic changes in cells membrane were examined using the flow cytometry method. Changes in Bax and Bcl2 gene expression were investigated using real-time PCR. The MIC and IC50 concentration of Daphne extract combine with Imatinib were tested on the K-562 cell line.

Results

The K-562 cells responded to extract treatments in a dose-dependent manner, and the MIC and the IC50 of the crude extract were calculated. The cell viability was quantitated by MTT assay.

Conclusion

Daphne extract could effectively decrease the viability of the cell line. The necrotic effect of Daphne extract was evaluated by Flow cytometry of Annexin, P.I., and an increase in gene expression of Bax and Bcl2 was observed in cells exposed to the Daphne extract. The combination of Daphne extracts with Imatinib shows enhancement in the cytotoxic effect of Imatinib.

Introduction

Plant natural products are a valuable source of therapeutic agents with a wide range of mechanisms of action [1]. Daphne Mucronata Royle is a wild plant of the Thymelaeaceae family, distributed in several regions of Iran, and popularly, it is known as Kheweshk in Kermanshah province [2]. It has been used in the treatment of skin disorders traditionally[2]. Literature search reveals that different species of Daphne, such as mezereum, genkawa, olidoies, and odora have an excellent antimicrobial effect [3].

Mazarine component of Daphne mezereum has shown cytotoxic activity, Odoricin is a new nematocidal compound from Daphne odora, and daphnetin-8-glycoside from Daphne acuminate possesses cardiotoxic activity [4]. Some species of the genus Thymelaeaceae have long been used in traditional medicine in various countries like China, Iran, and Pakistan as valuable remedies and have been increasingly used as a pain reliever. Some active compounds of the Daphne genus have been isolated and identified [5].

The first targeted therapy in CML was Imatinib, which was marketed under the Gleevec brand and led to many advances in the treatment of patients with CML and Increased overall survival so, most patients continued to live up to 5 years without any clinical complications [6]. This drug can be considered as the first targeted therapy in leukemia [6]. The protein from the combination of ABL and BCR genes causes the production of a protein called tyrosine kinase, which activates a number of intracellular proteins that are associated with increased cell proliferation [6]. Imatinib has an inhibitory effect on tyrosine kinase and binds specifically to tyrosine kinase encoded by the BCR-ABL gene. The cancer cells that have been injected with this drug no longer are able to survive and multiply, and they thereby undergo apoptosis [7]. Imatinib keeps the disease in the dormant phase for several years, but mortality from chronic myelogenous leukemia is still high [8]. This study tries to increase the effectiveness of Imatinib by examining the effect of Daphne extract and its combination with Imatinib and investigating the intracellular mechanism of Daphne cytotoxic effect. This study has evaluated the cytotoxic effects of Daphne extract on K-562 cell line. To understand the mechanisms of cytotoxicity, Flow cytometry of Annexin, P.I. and the changes in gene expression of proteins involved in the apoptosis cascade, including BCL2 and Bax, were also evaluated.

Apoptosis is called programmed cell death, and different proteins are involved in the process of cell death [8]. Some of these proteins prevent cell death by increasing the amount, and others induce cell death. Bcl2 is a protein that has an anti-apoptotic role, and Bax is a pro-apoptotic protein [8].

Materials And Methods

Plant material

Daphne shrubs grow in the foothills of the Zagros Mountains in Kermanshah province. Daphne's leaves were collected from shrubs in June (2). The identification feature of this plant this month is small white flowers, and Herbarium Botanists of Tehran University approved this plant.

Preparation of plant extract

The leaves of this plant were separated from the stem and dried in the shade, and then powdered. The powdered dried leaves (500 g) of the plant were soaking in pure dichloromethane (CH_2Cl_2) for 24 h at Room temperature. The whole extract was filtered, and the solvent was evaporated under reduced pressure at 40–45◦C, then extracted using a soxhlet extractor for 48 h. The extract was filtered and

concentrated under reduced pressure in a Rotary Flash Evaporator, which yielded 2% (10 g) of dry extract [3]. The extracts were stored at 4◦C until analysis.

Reagents and chemicals

RPMI-1640(Sigma- Germany), Fetal Bovine Serum [FBS] (Gibco, Germany), streptomycin and penicillin (Gibco, Germany), dimethyl sulfoxide [DMSO] (Sigma, USA), cryovial, T25 & T75 (jetbiofil), centrifuge (Eppendorf), the fluorescent probe Annexin, propidium iodide (P.I.), Silica gel 60 (230–400 mesh)for column chromatography and TLC silica gel 60 F254from Merck (Darmstadt, Germany); cDNA (CinnaGen), Real-time PCR (Amplicon), RNA RNX plus (CinnaGen), 2X PCR Master Mix (EmeraldAmp MAX PCR), Ladder (50 bp) Fermentas.

Purified compound

The residue was fractionated on a silica gel column (40x 1.5 cm) using diethyl ether: chloroform mixture (8:2, 6:4 and finally 4:6, v/v) as the eluting solvents, into three fractions. The active component was purified from the second fraction using TLC techniques. The molecular weight of the purified compound was 662 mass units, using FAB/MS. The purity of the isolated compound has been confirmed by HPLC [5].

Cell cultures and treatment agent

The human leukemic cell line K562 was obtained from Pasteur Institute (Tehran, Iran) and maintained in RPMI-1640 medium with 10% v/v fetal bovine serum and 100 u/ml penicillin and 100 mg/ml streptomycin at 37◦C in a humidified atmosphere of 5% CO2and 95% of air. About 5×103 K562 cells were seeded in each well of a 96-microwell plate and treated with various concentrations of extract of Daphne, and the cultured cells were sub-cultured twice a week.

Analysis of cell viability and apoptosis

Cell viability was determined by MTT assay [9]. K562 cells (5 ×103 cells/well) were seeded to a 96-well culture plate and cultured with and without extract and curcumin taken as standard (20ug/ml) for 24 hr. At the end of treatment, 20 ul of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional three h at 37 ◦C. Supernatants of wells were taken out, the formazan crystals were dissolved in 100 ul of DMSO, and the Optical density values (O.D. absorption) were read at 570 nm using a multi-well plate reader (Statfax 2000, Awareness). Percent inhibition of proliferation was calculated as a fraction of control (without extract). Cell viabilities were evaluated by the following equation [10].

Viability (%) = 100 × (OD sample – OD blank) / (OD negative control – OD blank).

Minimum Inhibitory Concentration and IC50

The cytotoxicity of Daphne extract was evaluated by MTT assay so, MIC and IC50 was calculated using Graph Pad Software (Graph Pad Prism version 5 software) with three replicates for each concentration of Daphne extract. The stock solution of extract was prepared at 0.5 mg/ml in DMSO and kept at – 20◦C.

Flow cytometry Technique

Propidium Iodide and Annexin V-FITC dyes were used to evaluate apoptosis and necrosis using the flow cytometry technique [10]. In cells undergoing apoptosis, phospholipids increase in the outer layer of the membrane and bind to Annexin and are detectable by green fluorescence. P.I. cannot cross the membrane of healthy cells and binds to DNA in dying cells (apoptosis or necrosis) and is detectable by red fluorescence. In this method, healthy cells do not stain, and apoptotic cells get both colors, and necrotic cells only get P.I. color [11].

RNA Extraction

Total RNA was extracted using TRIZOL (Invitrogen, USA) reagent, according to the protocols. For RT-PCR analysis of both gene expression and quality, the NanoDrop UV-VIS 2000C spectrophotometer (THERMO, USA) was determined. RNA quality parameters recommended for RT-PCR analysis include UV SPECTROSCOPY A260/280 ratio of 1.8–2.0 and A260/A230 ratio greater than 1.8, 18s/28s rRNA ratio of 1.8–2.1 [12].

cDNA Synthesis and Real-Time PCR (RT-PCR)

After treatment with Daphne mucronate extract, cDNA was determined using the SYBERGREEN q RT-PCR kit (Invitrogen, USA) according to the protocols from the total RNA. PCR reaction was performed using reverse primers and forward primer for each gene (Takapouzist, Tehran, Iran). The β-actin as a housekeeping gene was used to normalize the cDNA variation. The sequence of forwarding and reverse primers for PCR was designed based on previous studies. Primer sequence for training: BAX forward primer: 5-$G\mathbb{C}$; BAX reverse primer: 5- TCCAATGTCCAGCCTTTG-3; BCL-2 forward primer: 5-CGGAGGCTGGGATGCCTTTG-3; BCL2 reverse primer: 5 TTTGGGGCAGGCATGTTGAC3 [13]. All reactions were triplicated.

Results

Determination of MIC and IC50

The anti-proliferative effects of Daphne mucronata on cancer cell lines K562 were evaluated by MTT assay. A dose-dependent decrease in the growth of cancer cells was observed with increasing concentrations (Fig. 1). The minimum inhibitory concentration is the concentration of the extract that prevents the increase in the number of cells after being added to the cells for a specified period. In the control sample, an increase in the number of cells was observed after 24 hours. At a concentration of 500 ng, the number of cells did not increase after 24 hours. IC50 is a concentration of Daphne extract that reduces the cell population to half after a specific time, so 7ug/ml is the IC50 of the daphne extract.

Microscope examination

Morphologic change of K-562 cells after treatment with daphne extract analyzed under a microscope. Pyknotic change after 24 hr has been seen in the number of cells, and ruptured cell has been seen after 48 hr. Apoptosis or programmed cell death is recognized by characteristic morphological and molecular changes occurring in a cell [9]. To evaluate the cause of growth inhibition of K562 cells by Daphne extract, characteristic features of morphological apoptosis were studied. Daphne extract-treated cells showed prominent morphological changes like cell shrinkage with rounding of cells and formation of membrane blebs characteristic of apoptosis as evidenced by microscopic studies (Fig. 2).

Combination Imatinib and Daphne extract

The concentration of Imatinib, which can reduce viable cell population to 50% after 24 hours, was calculated. Then, this concentration of Imatinib combined with different concentrations of Daphne extract on K-562 cells (Fig. 3). The results show that this combination of imatinib and daphne extract changes significantly in the cell population. The Anti-proliferative effect of Imatinib could increase in the mix of Daphne extract.

Flow cytometry results

Flow cytometry assay results show that cells at different concentrations of Daphne extract were PI-positive and Annexin negative, which was an indicator of necrotic cells (Fig. 4). Combination treats of K-562 cell line with Imatinib, and Daphne extract show that some populations of cells were apoptotic, and some populations were necrotic. The rate of necrosis in cells increases with concentration (Fig. 5).

Bax and Bcl-2 Real-time PCR

Bax proteins possess a crucial function in controlling cytochrome C release and apoptosis initiation via the mitochondrial pathway [12]. The vast majority of K562 cells in the untreated control were healthy. By contrast, treatment by Daphne extracts resulted in marked necrotic induction in a dose-dependent manner (Fig. 6). Treatment with Daphne extract that blocks cell division led to a change in Bax and Bcl-2 gene expression. The ratio of Bax to Bcl-2 acts as an indicator for the cell apoptosis pathway (Fig. 7). Bax/Bcl-2 Ratio could clearly show the cell's tendency to apoptosis pathway, so an increase in Bax/Bcl-2 ratio conduct cell to apoptosis, and a decrease in this ratio causes cell resistance to apoptosis [13].

Discussion

The plant extract and the purified active component of Daphne extract could inhibit the proliferation of the K-562 cell lines [5]. Previous studies investigated the effects of Daphne mucronata extract to establish the scientific validation and understand the molecular mechanism behind these folk claims [2]. This study investigated the effect of this extract on cell growth and death mechanisms in the K-562 cell line.

Effect of Daphne mucronata on cancer cell lines K562 was carried by MTT assay. 500ng/ml concentration of Daphne extract can inhibit cell proliferation. Daphne Extract can reduce cell viability to half (IC50) at 7ug/ml concentration on K562 cell lines. Hence further studies to confirm molecular

mechanisms were carried on K562 cells. One of the biochemical features of apoptotic cells is the expression of cell surface markers achieved by flip-flop movement of the P.S. from the inner membrane to the outer membrane of the plasma membrane [11]. Results from flow cytometry assay demonstrated that the Annexin and P.I. positive cells indicated induction of apoptosis by Imatinib [11]. On the other hand, P.I. positive cell indicated induction of necrosis by the Daphne extract [11]. This study also explored the underlying molecular events occurring due to Daphne extract treatment. Increase gene expression of Bax and reduction of Bcl-2 is an expected outcome from the apoptosis process [13]. K-562 cell line that was treated with Imatinib shows these changes in gene expression that indicate the apoptosis effect of Imatinib on the K-562 cell line. But K-562 cell that was treated with Daphne shows an increase in Bax and Bcl-2 gene expression. Although flow cytometry result indicates and necrosis effect of Daphne extract, so increase in gene expression of Bax along with Bcl-2 could demonstrate a necrosis prosses. It has been previously thought that necrosis is an alternative process of cell death [7]. Recent studies suggest that necrosis is a planned cell death process is one of the genes that increase its expression in both apoptosis and necrosis in the Bax gene [7]. However, Bcl2 gene expression decreases in the process of apoptosis and should increase in necrosis. The current study clearly indicates that Daphne extract could act against K-562 cell line. Daphne extract decreases the cell viability after combination with Imatinib. The results of this study showed that the extract obtained from Herbal Medicine could have a synergistic effect with chemotherapy drugs on K-562-cell line. Further studies are needed to evaluate the effect of this compound clinically. The natural plant component has a lower clinical indication in comparison to the chemical component [14]. Daily consumption of Imatinib in CML patients can cause resistance to Imatinib after five years, and many patients die after the recurrence of the disease [8]. Combination treatment of patients would be an effective method to overcome resistance and reduce daily Imatinib consumption dose for CML patients.

Conclusion

Herbal remedies used in traditional medicine are still used in some communities. New research has tried to find the cause of the beneficial effects of these old remedies. Imatinib is a tyrosine kinase inhibitor that uses for target therapy of chronic myelogenous leukemia. Many studies have shown that the median survival rate of CML patients with Imatinib treatment is five years. Patients died after the recurrence of the disease, because they became resistant to Imatinib. Results of this in-vitro study show that daphne extract has an anti-proliferative effect on the K-562 cell line. A combination of daphne extract and Imatinib in vitro shows an excess reduction in the cell population. However, Daphne extract cause cell to became necrosis but this necrosis process cause change in gene expression. Patterns of gene expression were different in cells treated with daphne extract in comparison to Imatinib treatment. Further studies are needed for clinically evaluation of combine treatment of patients with Imatinib and daphne.

Declarations

Ethics approval and consent to participate

All methods were carried out in accordance with the relevant institutional, national, and international guidelines and legislation. Besides they were discussed and approved by the Research Ethics Committee of Tarbiat Modares University.The samples were picked from mature plants, put in labeled sealed plastic bags, and stored at 4 °C until preparation for extraction.

Permissions or licenses to collect different parts of the plants were obtained from owners. All stages of plant finding, and species determination and identification were performed under the supervision of Dr. Amir Hossein Asghari based on Colorful Flora of Iran, and confirmed by Dr Vali-allah Mozaffarian of the Herbarium Institute of Tehran University,Tehran, Iran and a voucher specimen (ID: 6537) deposited at the herbarium of the institute.

In addition, this study, use of the K-562 cell lines, was approved by Medical Ethics Committee, Faculty of Medical Sciences, Tarbiat Modares University, on April 28, 2017; approval reference number: IR.TMU.TEC.1395.5.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

During research and preparing the manuscript Alireza Khorshid performed the primitive research, study, classified and noted the available articles, prepared the primitive manuscripts and Javid Sabour Takanlu and Jafar Nouri Nojadeh participated in the general idea of the study, preparing the Figures and final editing, and submitting the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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Figures

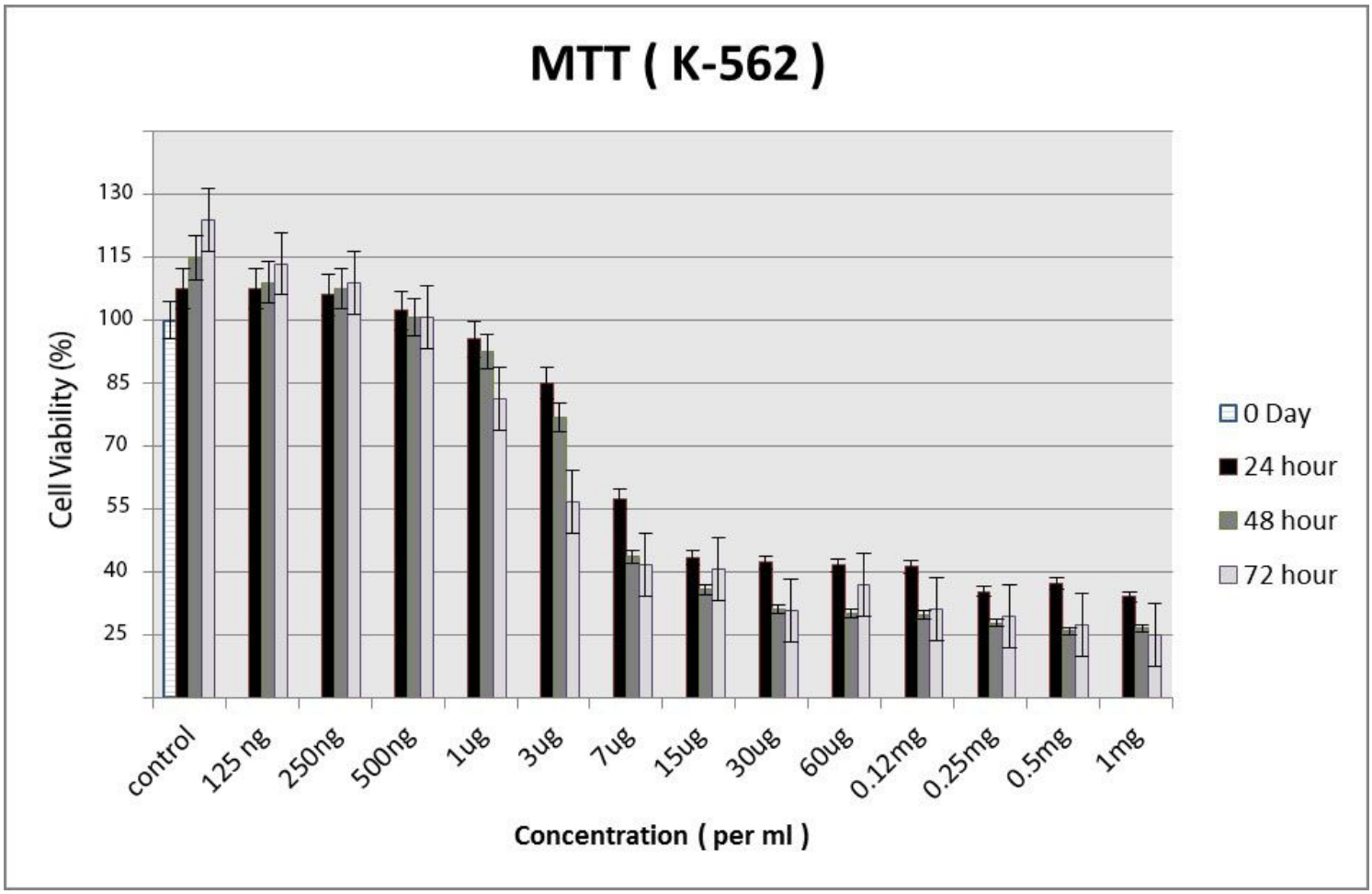

Figure 1

MTT assay result with a percentage of cell viability.

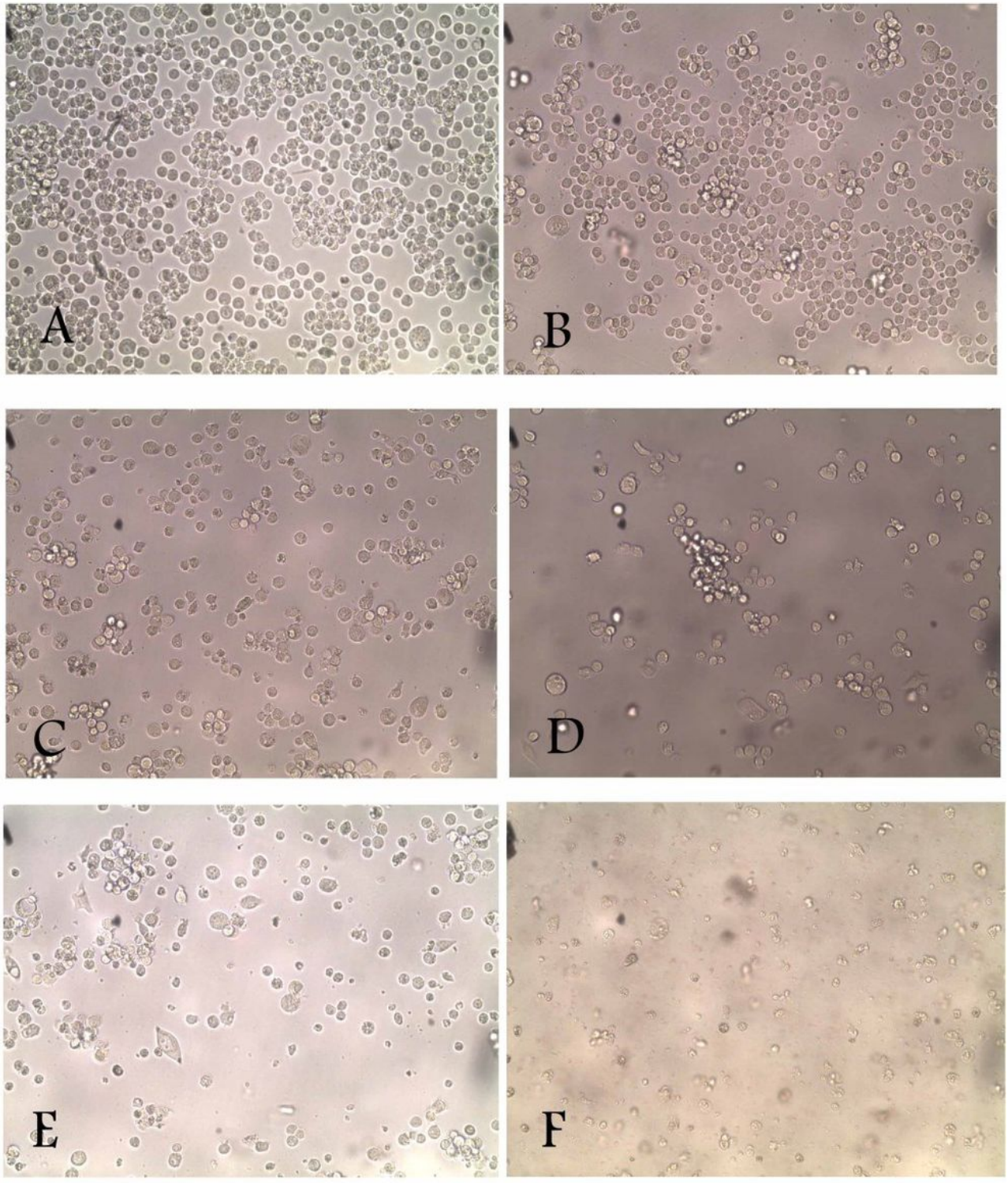

Figure 2

Morphological change 24hr and 48hr after treatment with daphne extract. Normal K-562 Cell-line before treatment (A). Cells 24hr after Incubation (B). 24hr after treatment with 500 ng/ml extract (C). 28hr after treatment with 500ng/ml extract (D). 24hr after treatment with 7ug/ml extract (E). 48hr after treatment with 7ug/ml extract (F).

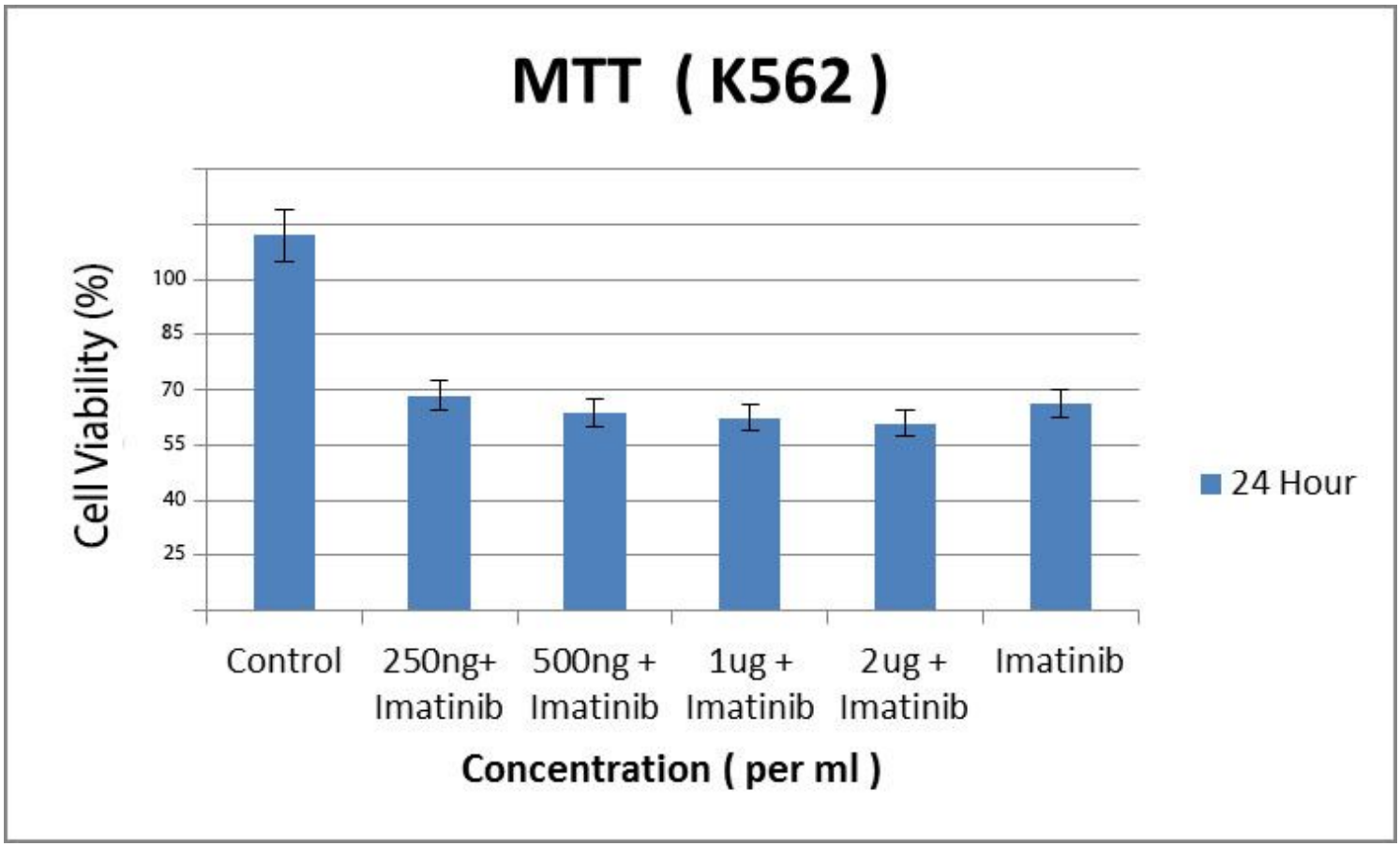

Figure 3

Combine effect of Imatinib and daphne extract on K-562 cell line 24 hrs. after treatment.

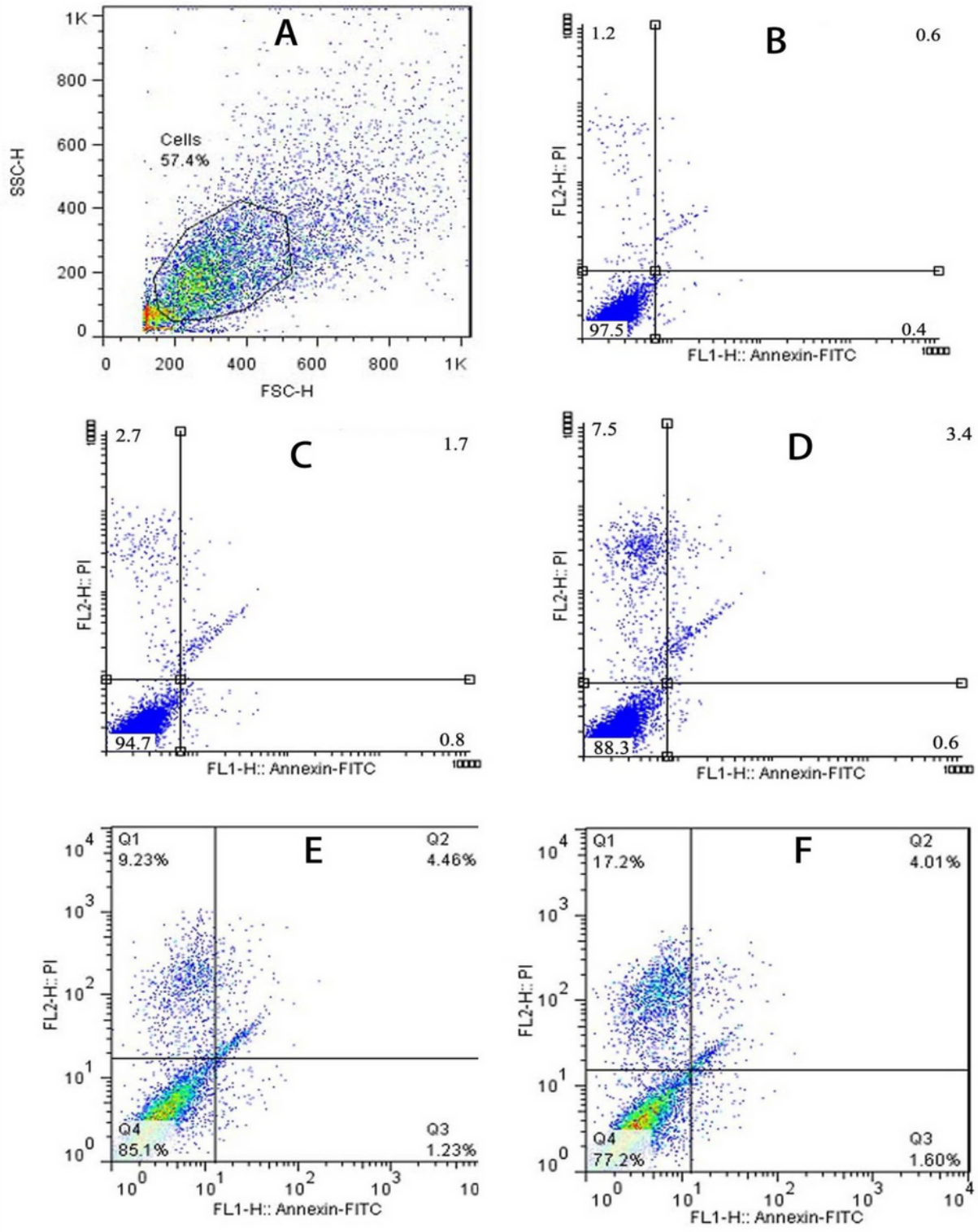

Figure 4

Effect of treatment of K-562 cell line with different concentrations of Daphne extract after 24hr. Gating of the cell population (A). K-562 without treatment – Normal Control (B). Treatment with 125ng/ml Daphne extract (C). Treatment with 256 ng/ml Daphne extract (D). Treatment with 500ug/ml Daphne extract (E). Treatment with 1ug/ml Daphne extract (F).

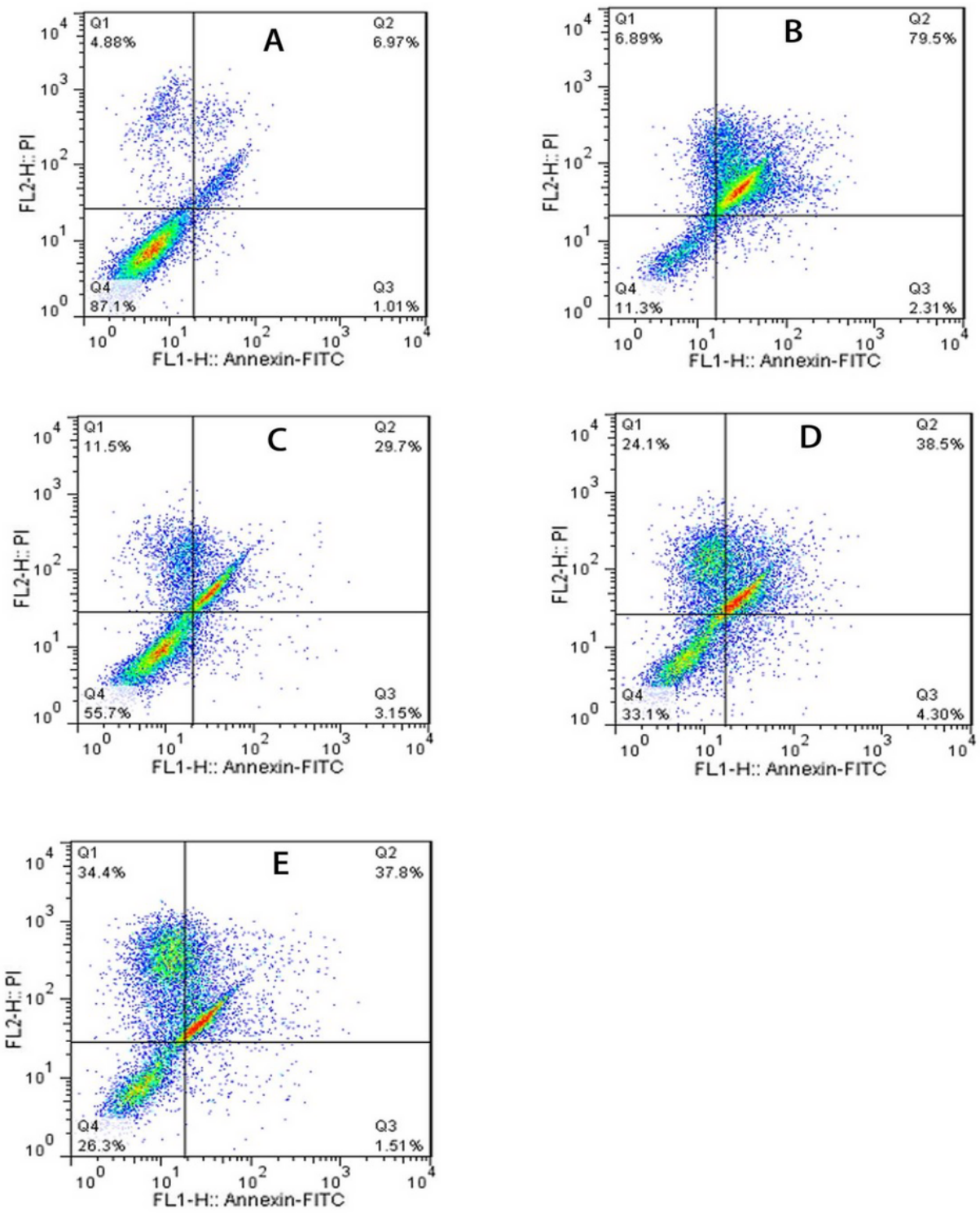

Figure 5

Combination of 1uM Imatinib with different concentrations of Daphne extract on K-562 cell line after 24h.
A control population of cell line(A). Imatinib (1uM) treatment (B). Combination of Imatinib with 500ng/ml

Daphne extract (C). Combination of Imatinib with 1ug/ml Daphne extract (D). Combination of Imatinib with 3ug/ml Daphne extract (E).

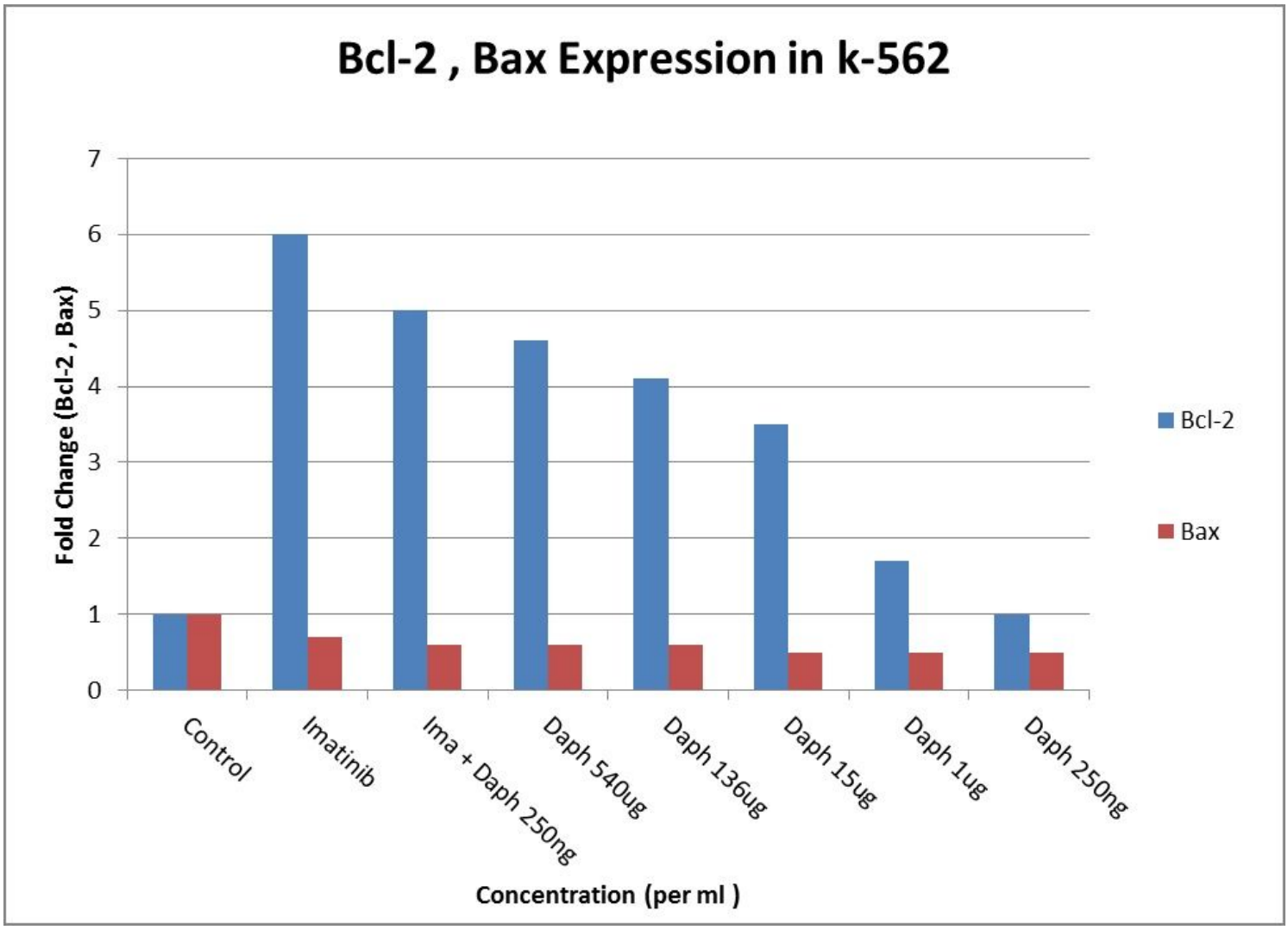

Figure 6

Bax and Bcl-2 change in gene expression after treatment with Imatinib and Daphne Mucronata.

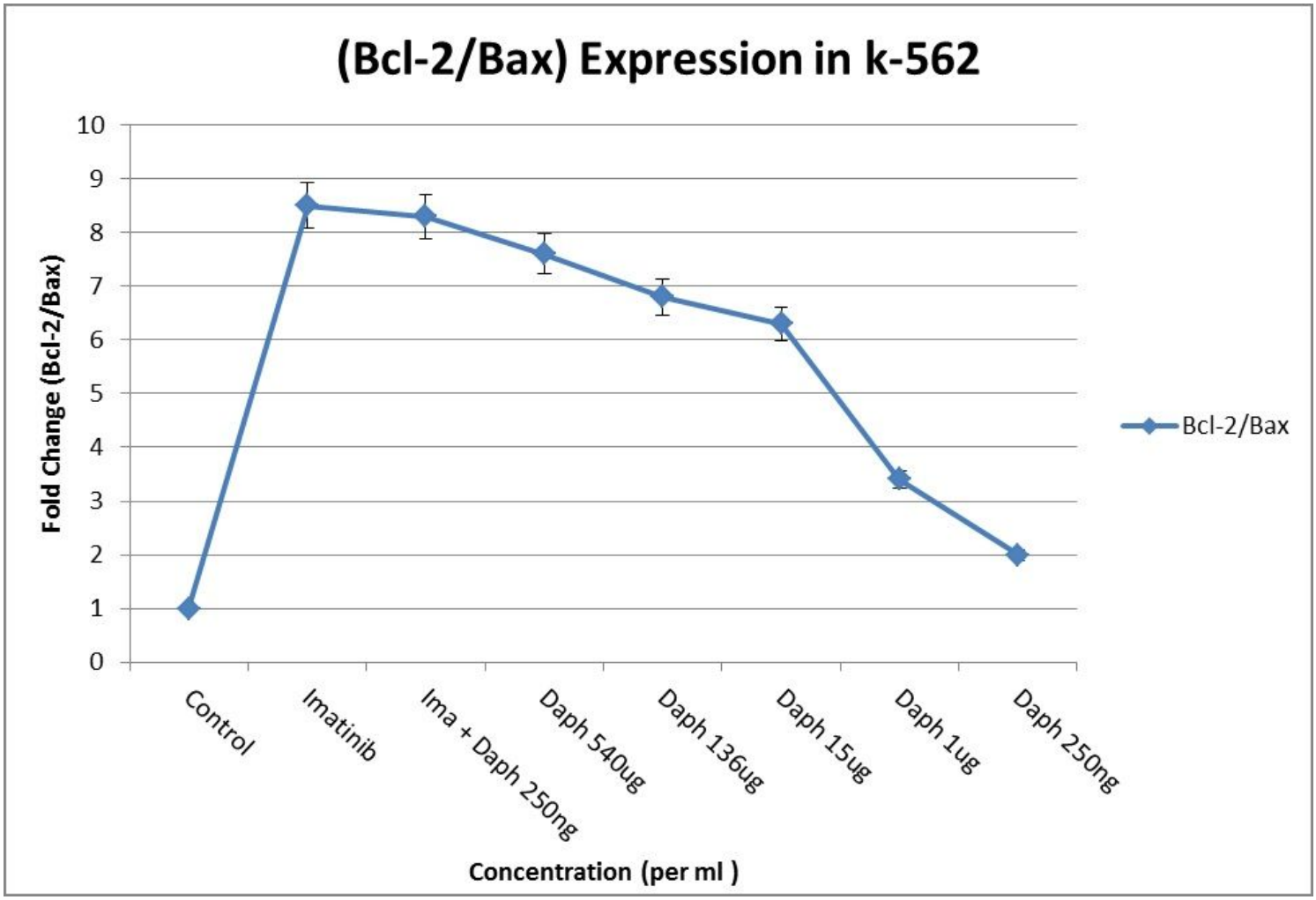

Figure 7

Bax to Bcl-2 ratio in K-562 cell after treatment with Imatinib and different concentrations of Daphne extract.