

The Combination Effect of Daphne Extract and Imatinib on the Antiproliferative Activity of the K562 Cell Line

Alireza Khorshid

Ali.r.khorshid@gmail.com

University of Texas Medical Branch <https://orcid.org/0000-0003-0345-1862>

Jafar Nouri Nojadeh

Tabriz University of Medical Sciences

Javid Sabour Takanlu

jsabour77@gmail.com

National University of Ireland Galway

Research Article

Keywords: Daphne, Imatinib, apoptosis, herbal medicine, Bax, Bcl-2, CML

Posted Date: July 9th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4694814/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: The authors declare no competing interests.

Abstract

Background: 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 K562 cell line has been previously studied, and Gleevec is a well-known and effective drug for the treatment of chronic myelogenous leukemia.

Material and Methods: In this study, the simultaneous effects of using herbal medicine and a targeted therapy drug on the K562 cell line were investigated. The presence of some species of *Daphne* in Iran motivated us to evaluate the cytotoxic effect of *Daphne mucronata* on human leukemia cancer cells. The antiproliferative activity of the dichloromethane extract of *daphne mucronate* (Thymelaeaceae), a new anticancer medicinal plant, was evaluated. Cell viability was quantitated by MTT assay. Apoptotic and necrotic changes in the cell membrane were examined using flow cytometry. Changes in Bax and Bcl2 gene expression were investigated using real-time PCR. The MIC and the IC50 of the crude extract were calculated, and the MIC and IC50 of the combination of the *Daphne* extract and imatinib were tested in the K-562 cell line.

Results: K-562 cells responded to the extract treatments in a dose-dependent manner, and the increase in the expression of Bcl2 and decrease in the expression of the Bax gene increased with increasing extract concentration. Flow cytometry revealed that most of the cells underwent apoptosis.

Conclusion: *Daphne* extract effectively decreased the viability of the cell line. The necrotic effect of the *Daphne* extract was evaluated, and an increase in the gene expression of Bcl2 was observed in cells exposed to the *Daphne* extract. The combination of *Daphne* extracts with imatinib enhances the cytotoxic effect of imatinib.

Introduction

Plant natural products are valuable 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 traditional treatment of skin disorders (2). A literature search revealed that different species of *Daphne*, such as *mezereum*, *genkawa*, *olidoies*, and *odora*, have excellent antimicrobial effects (3).

The azarine component of *Daphne mezereum* has shown cytotoxic activity, Odoricin is a new nematocidal compound from *Daphne odora*, and the daphnetin-8-glycoside from *Daphne acuminata* has cardiotoxic activity (4). Several species of the genus Thymelaeaceae have long been used in traditional medicine in various countries, such as China, Iran, and Pakistan, as valuable remedies and have been increasingly used as pain relievers. Several active compounds of the *Daphne* genus have been isolated and identified (5). The use of plant extracts and natural substances to develop effective drugs for cancer treatment is increasing (6). The cytotoxic effects of plant extracts on cancer cells are significant (6). Medicines made from natural materials and herbal extracts have been shown to reduce drug resistance

and increase survival in patients (7). The incidence of multidrug resistance is increasing in leukemia patients, and the combination of modern treatment with traditional medicine has been shown to delay drug resistance (7). Leaf extracts of *Daphne* plants have effects on growth inhibition and cell cycle inhibition in acute myeloblastic leukemia cell lines (8). Gnidilatimonoein (Gn) is a diterpene obtained from the leaf extract of *Daphne macronata* plants that has cytotoxic properties (9).

The first targeted therapy for 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; thus, most patients continue to live for up to 5 years without any clinical complications (10). This drug can be considered the first targeted therapy for leukemia. The protein produced by the combination of the ABL and BCR genes results in the production of a protein called tyrosine kinase, which activates a number of intracellular proteins that are associated with increased cell proliferation (10). Imatinib has an inhibitory effect on tyrosine kinases and binds specifically to tyrosine kinases encoded by the BCR-ABL gene. Cancer cells that have been injected with this drug are no longer able to survive or multiply, and they thereby undergo apoptosis (11). In addition, imatinib keeps the disease in the dormant phase for several years, but mortality from chronic myelogenous leukemia is still high (12). This study aimed to increase the effectiveness of imatinib by examining the effect of *Daphne* extract alone or in combination with imatinib and investigating the intracellular mechanism of the cytotoxic effect of *Daphne*. This study evaluated the cytotoxic effects of *Daphne* extract on chronic myelogenous leukemia cells. To understand the mechanisms of cytotoxicity, flow cytometry (FCM) analysis of Annexin and P.I. and changes in the 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 this process (12). Some of these proteins prevent cell death by increasing the amount of protein needed, while others induce cell death. Bcl2 is a protein that has an antiapoptotic effect, and Bax is a proapoptotic protein (12).

Materials and methods

Plant material

Daphne shrubs grow in the foothills of the Zagros Mountains in Kermanshah Province near Paveh city. *Daphne* leaves were collected from shrubs in June 2015, and the *Daphne* plant has white and small flowers (2). The identification feature of this plant this month was small white flowers, and Herbarium Botanists of Tehran University approved this plant; the plant was registered in the Tehran University herbarium with voucher number QUETTA000158.

Preparation of plant extract

The leaves of these plants were separated from the stem, dried in the shade, and then powdered. The powdered dried leaves (500 g) of the plants were soaked 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 and 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) 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), commercial T25 and T75 (Jetfoil), centrifuge (Eppendorf), the fluorescent probes Annexin and propidium iodide (P.I.), silica gel 60 (230–400 mesh) for column chromatography and TLC silica gel 60 F254 from Merck (Darmstadt, Germany), cDNA (CinnaGen), real-time PCR (Amplicon), RNA RNX plus (CinnaGen), 2X PCR Master Mix (EmeraldAmp MAX PCR), and Ladder (50 bp) Fermentas were used.

Purified compound

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

Cell culture and treatment agents

The human leukemia cell line K562 was obtained from Pasteur Institute (Tehran, Iran) and maintained in RPMI-1640 medium supplemented with 10% v/v fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Approximately 5×10³ K562 cells were seeded in each well of a 96-well microplate and treated with various concentrations of Daphne extract, after which the cultured cells were subculture twice a week.

Analysis of cell viability and apoptosis

Cell viability was determined by MTT assay (13). K562 cells (5 ×10³ cells/well) were seeded in a 96-well culture plate and cultured with or without extract or curcumin (20 µg/ml) for 24, 48 or 72 hr. Plant extracts were prepared at various concentrations (1 mg, 0.5 mg, 0.25 mg, 0.12 mg, 60 µg, 30 µg, 15 µg, 7 µg, 3 µg, 1 µg, 500 ng, 250 ng, and 125 ng). At the end of treatment, 20 µl of MTT (5 mg/ml in PBS) was added to each well, and the cells were incubated for an additional three hours at 37°C. The supernatants of the wells were removed, the formazan crystals were dissolved in 100 µl of DMSO, and the optical density (O.D. absorption) was read at 570 nm using a multicell plate reader (Statfax 2000, Awareness). The percent inhibition of proliferation was calculated as a fraction of the control (without extract). Cell viability was evaluated by the following Eq. (14):

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

Minimum inhibitory concentration and IC₅₀

The cytotoxicity of the daphne extract was evaluated by an MTT assay, and the MIC and IC₅₀ were calculated using GraphPad Prism version 5 software, with three replicates for each concentration of the

daphne extract. A stock solution of the extract was prepared at 0.5 mg/ml in DMSO and kept at – 20°C.

Flow cytometry technique

Propidium iodide (PI) and annexin V-FITC dyes were used to evaluate apoptosis and necrosis, respectively, via flow cytometry (14). In cells undergoing apoptosis, the phospholipid concentration increases in the outer layer of the membrane, and phospholipids bind to Annexin and are detectable by green fluorescence. P.I. cannot cross the membrane of healthy cells, bind to DNA in dying cells (apoptotic or necrotic) and is detectable by red fluorescence. In this method, healthy cells are not stained, apoptotic cells are stained, and necrotic cells are stained only by the P.I. (15).

RNA Extraction

Total RNA was extracted using Triazole (Invitrogen, USA) reagent according to the manufacturer's protocols. For RT–PCR analysis of both gene expression and quality, a NanoDrop UV–VIS 2000C spectrophotometer (Thermo Fisher Scientific, USA) was used. The RNA quality parameters recommended for RT–PCR analysis included a UV SPECTROSCOPY A260/280 ratio of 1.8-2.0, an A260/A230 ratio greater than 1.8, and an 18 s/28 s rRNA ratio of 1.8–2.1 (16).

cDNA synthesis and real-time PCR (RT–PCR)

After treatment with Daphne mucronate extract, cDNA expression was determined using the SYBERGREEN q RT–PCR Kit (Invitrogen, USA) according to the manufacturer's protocol for total RNA extraction. PCR was performed using reverse primers and forward primers for each gene (Takapouzist, Tehran, Iran). β -Actin was used as a housekeeping gene to normalize the cDNA variation. The sequences of the forward and reverse primers used for PCR were designed based on previous studies. The sequences of primers used for training were as follows: BAX forward primer: 5 – *GCC T T GC T CAGGG T T – C*; BAX reverse primer: 5 – *TC V TGT CAGC T TG – 3*; BCL-2 forward primer: 5 – *CGGAGGCTGGGATGC T TG – 3*; and BCL2 reverse primer: 5 – *TGGGGCAGGCATG T GAC* (17). All the reactions were performed in triplicate.

Results

Determination of MIC and IC50

The antiproliferative effects of Daphne mucronata on the K562 cancer cell line were evaluated by MTT assay. A dose-dependent decrease in the growth of cancer cells was observed with increasing concentrations. The minimum inhibitory concentration was the concentration of the extract that prevented an increase in the number of cells after the extract was added to the cells for a specified period of time. In the control sample, an increase in the number of cells was observed after 24 hours. After treatment with 500 ng of BQ, the number of cells did not increase after 24 hours. The IC50 is the concentration of Daphne extract that reduces the cell population to half after a specific time period, so 7 μ g/ml is the IC50 of the Daphne extract.

Microscope examination

Changes in the morphology of K-562 cells after treatment with Daphne extract were analyzed under a microscope. A pyknotic change in the number of cells was observed after 24 hr, and ruptured cells were observed after 48 hr. Apoptosis or programmed cell death is recognized by characteristic morphological and molecular changes occurring in a cell (13). To evaluate the cause of Daphne extract-induced growth inhibition in K562 cells, characteristic features of morphological apoptosis were studied. Daphne extract-treated cells exhibited prominent morphological changes, such as cell shrinkage, cell rounding and the 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 the viable cell population to 50% after 24 hours, was calculated. Then, K-562 cells were treated with different concentrations of imatinib in combination with Daphne extract. The results showed that the combination of imatinib and Daphne extract significantly changed the cell population. The antiproliferative effect of imatinib could increase when the mixture of Daphne extract was used.

Flow cytometry results

Flow cytometry assays revealed that cells treated with different concentrations of Daphne extract were PI positive and Annexin negative, which are indicators of necrotic cells. Combinations of treatment of the K-562 cell line with imatinib and Daphne extract showed that some populations of cells were apoptotic, while others were necrotic. The percentage of necrotic cells increased with increasing concentration (Fig. 5).

Bax and Bcl-2 real-time PCR

Bax proteins play a crucial role in controlling cytochrome C release and initiating apoptosis via the mitochondrial pathway (16). The vast majority of K562 cells in the untreated control group were healthy. In contrast, treatment with Daphne extracts resulted in marked necrotic induction in a dose-dependent manner. Treatment with Daphne extract, which blocks cell division, led to changes in Bax and Bcl-2 gene expression. The ratio of Bax to Bcl-2 acts as an indicator of the extent of cell apoptosis. The Bax/Bcl-2 ratio clearly reflects cell apoptosis pathway activity; an increase in the Bax/Bcl-2 ratio induces cell 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 K-562 cells (5). This study investigated the effects of Daphne mucronata extract to establish a scientific validation and understand the molecular mechanism underlying these folk claims.

The effect of Daphne mucronata on the K562 cancer cell line was evaluated via an MTT assay. A total of 500 ng/ml Daphne extract inhibited cell proliferation. Daphne extract reduced cell viability to half (IC₅₀)

at a concentration of 7 µg/ml in K562 cells. Hence, further studies to confirm the molecular mechanisms involved were carried out in 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 (15). The results from the flow cytometry assays demonstrated that the Annexin and P.I.-positive cells were induced to undergo apoptosis by imatinib. On the other hand, P.I.-positive cells exhibited necrosis induced by Daphne extract (15). This study also explored the underlying molecular events occurring due to Daphne extract treatment. An increase in the gene expression of Bax and a reduction in the expression of Bcl-2 are expected outcomes of apoptosis (13 K-562 cells treated with imatinib showed changes in gene expression that indicate the apoptotic effect of imatinib on the K-562 cell line). However, K-562 cells treated with Daphne showed an increase in Bax and Bcl-2 gene expression. Although the flow cytometry results indicated the necrosis effect of Daphne extract, the increase in the gene expression of Bax and Bcl-2 could indicate necrosis. It was previously thought that necrosis is an alternative process of cell death. Recent studies suggest that necrosis is a planned cell death process—one of the genes that increases the expression of both apoptotic and necrotic genes in the Bax gene. However, Bcl2 gene expression decreases during the process of apoptosis and should increase during necrosis. The current study clearly indicated that Daphne extract could be a potent antileukemic agent against chronic myeloid leukemia cells and the K-562 cell line. Daphne extract decreases cell viability after combination with imatinib. Therefore, the efficacy of imatinib in the treatment of CML could be improved by combination with Daphne extract. The natural plant component has a lower clinical indication than the chemical component. Daily consumption of imatinib in CML patients can cause resistance to imatinib after five years, and many patients die after disease recurrence (12). Patient combination therapy could be an effective method for overcoming resistance and reducing the daily intake of imatinib in CML patients.

Conclusion

Imatinib is a tyrosine kinase inhibitor that is used for targeted therapy for chronic myelogenous leukemia. The median survival rate of CML patients treated with imatinib is five years. However, many patients die after disease recurs and become resistant to imatinib. The results of this study showed that Daphne extract has an antiproliferative effect on the K-562 cell line. A combination of Daphne extract and imatinib results in an excess reduction in the cell population. However, Daphne extract caused necrosis, but this necrosis process caused changes in gene expression. The patterns of gene expression differed between cells treated with Daphne extract and those treated with imatinib. Combined treatment of patients with imatinib and daphne can increase patient survival and decrease resistance to imatinib.

Declarations

Acknowledgments

Not applicable.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

During the research and preparation of the manuscript, Alireza Khoshid performed the primitive research and study, classified and noted the available articles, and prepared the primitive manuscripts. Javid Sabour Takanlu and Jafar Nouri Nojadeh participated in the general idea of the study, prepared the figures and finally edited and submitted the manuscript. All the authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests

The authors state that they have no conflicts of interest.

Informed consent

Informed consent was obtained from all individuals included in this study.

Ethical approval

The local Institutional Review Board deemed the study exempt from review.

References

1. Mahdizadeh S, Ghadiri MK, Gorji A. Avicenna's Canon of Medicine: a review of analgesics and anti-inflammatory substances. *Avicenna journal of phytomedicine*. 2015;5(3):182.
2. Javidnia K, Miri R, BAHRI NR, KHADEMZADEH JN. A preliminary study on the biological activity of *Daphne mucronata* Royle. 2003.
3. Kupchan SM, Baxter RL. Mezerein: antileukemic principle isolated from *Daphne mezereum* L. *Science*. 1975;187(4177):652-3.
4. Nasipuri R, Ramstad E. Isolation of Daphnetin-8- β -glucoside from *Daphne Papyracea*. *Journal of Pharmaceutical Sciences*. 1973;62(8):1359-60.
5. L Liou Y, Hall I, Lee K. Antitumor agents LVI: The protein synthesis inhibition by genkwadaphnin and yuanhuacine of P-388 lymphocytic leukemia cells. *Journal of Pharmaceutical Sciences*. 1982;71(12):1340-4.
6. Maher T, Ahmad Raus R, Daddiouaissa D, et al. Medicinal Plants with Anti-Leukemic Effects: A Review. *Molecules*. 2021;26(9):2741. Published 2021 May 7. doi:10.3390/molecules26092741
7. Senthilkumar R, Chen BA, Cai XH, Fu R. Anticancer and multidrug-resistance reversing potential of traditional medicinal plants and their bioactive compounds in leukemia cell lines. *Chin J Nat Med*.

- 2014 Dec;12(12):881-94. doi: 10.1016/S1875-5364(14)60131-X. PMID: 25556059.
8. Nouri K, Yazdanparast R. Proliferation inhibition, cell cycle arrest and apoptosis induced in HL-60 cells by a natural diterpene ester from *Daphne mucronata*. *Daru*. 2011;19(2):145-153.
 9. Mianabadi M, Rivera E, Yazdanparast R. A new diterpene ester from the leaves of *Daphne mucronata*. *J Trop Medl Plan*. 2003; 4:1-4.
 10. Sacha T. Imatinib in chronic myeloid leukemia: an overview. *Mediterranean journal of hematology and infectious diseases*. 2014;6(1).
 11. G Ghanadian M, Ali Z, Khan IA, Balachandran P, Nikahd M, Aghaei M, et al. A new sesquiterpenoid from the shoots of Iranian *Daphne mucronata* Royle with selective inhibition of STAT3 and Smad3/4 cancer-related signaling pathways. *DARU Journal of Pharmaceutical Sciences*. 2020;28(1):253-62.
 12. Bhamidipati PK, Kantarjian H, Cortes J, Cornelison AM, Jabbour E. Management of imatinibresistant patients with chronic myeloid leukemia. *Therapeutic advances in hematology*. 2013;4(2):103- 117.
 13. Elmore S. Apoptosis: a review of programmed cell death. *Toxicologic pathology*. 2007;35(4):495-516.
 14. Paudel MR, Chand MB, Pant B, Pant B. Cytotoxic activity of antioxidant-riched *Dendrobium longicornu*. *Pharmacognosy Journal*. 2017;9(4).
 15. R Rieger AM, Nelson KL, Konowalchuk JD, Barreda DR. Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. *JoVE (Journal of Visualized Experiments)*. 2011 (50):e2597.
 16. Tan SC, Yiap BC. *J. Biomed. Biotechnol*. 2009.
 17. Brock K, Talley K, Coley K, Kundrotas P, Alexov E. Optimization of electrostatic interactions in protein–protein complexes. *Biophysical journal*. 2007;93 (10):3340-3352.

Figures

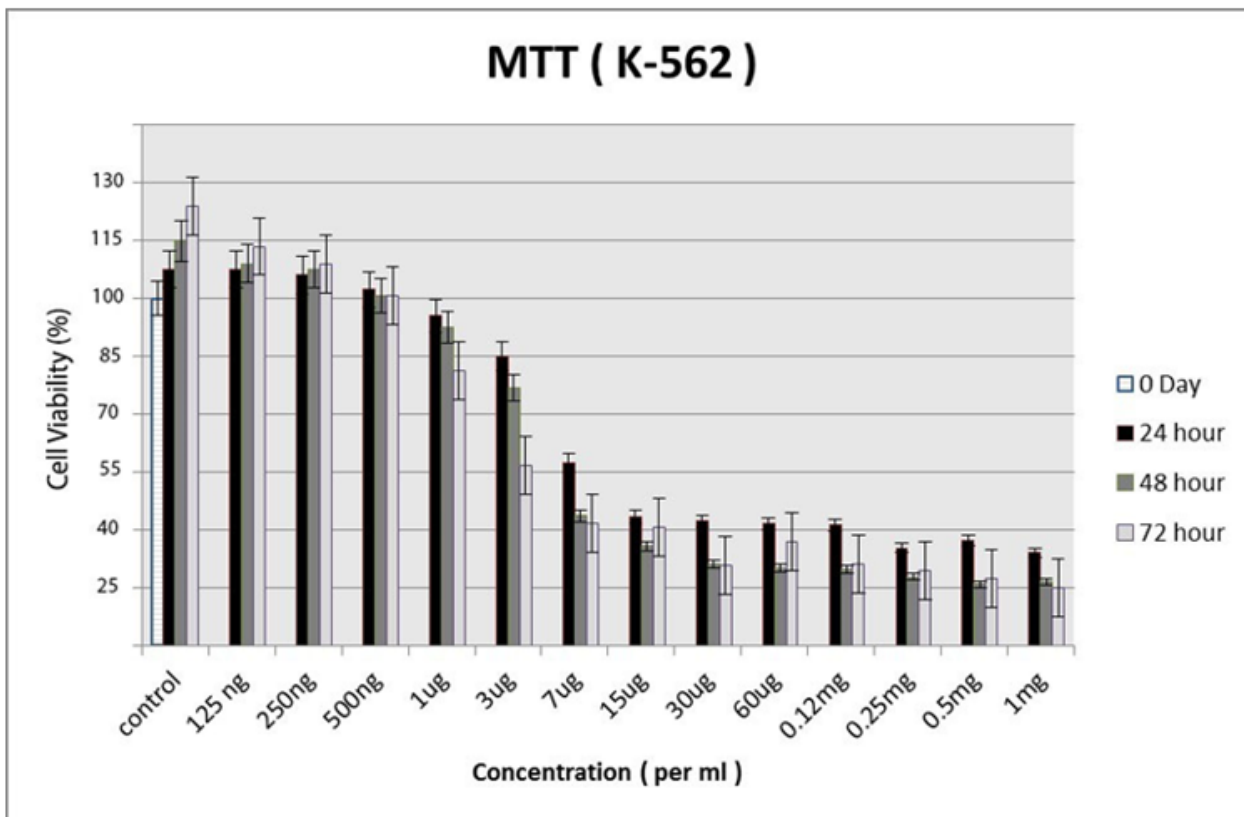


Figure 1

The MTT assay was used to determine the percentage of cell viability.

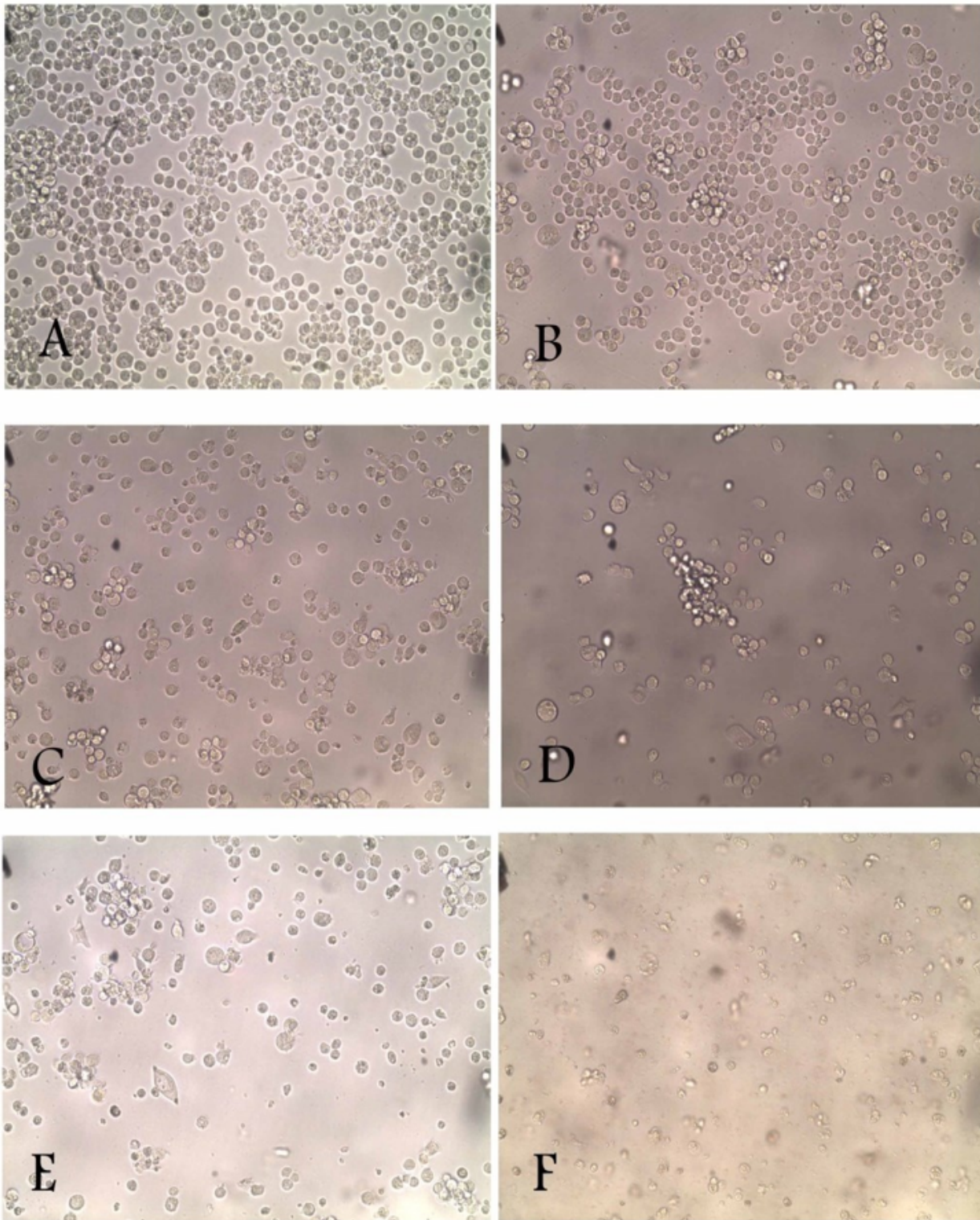


Figure 2

Morphological changes 24 hr and 48 hr after treatment with Daphneextract. Normal K562 cell line before treatment (A). Cells 24 hr after incubation (B). 24 hr after treatment with 500 ng/ml extract (C). 48 hr after treatment with 500 ng/ml extract (D). Twenty-four hours after treatment with 7 µg/ml extract (E). 48 hr after treatment with 7 µg/ml extract (F).

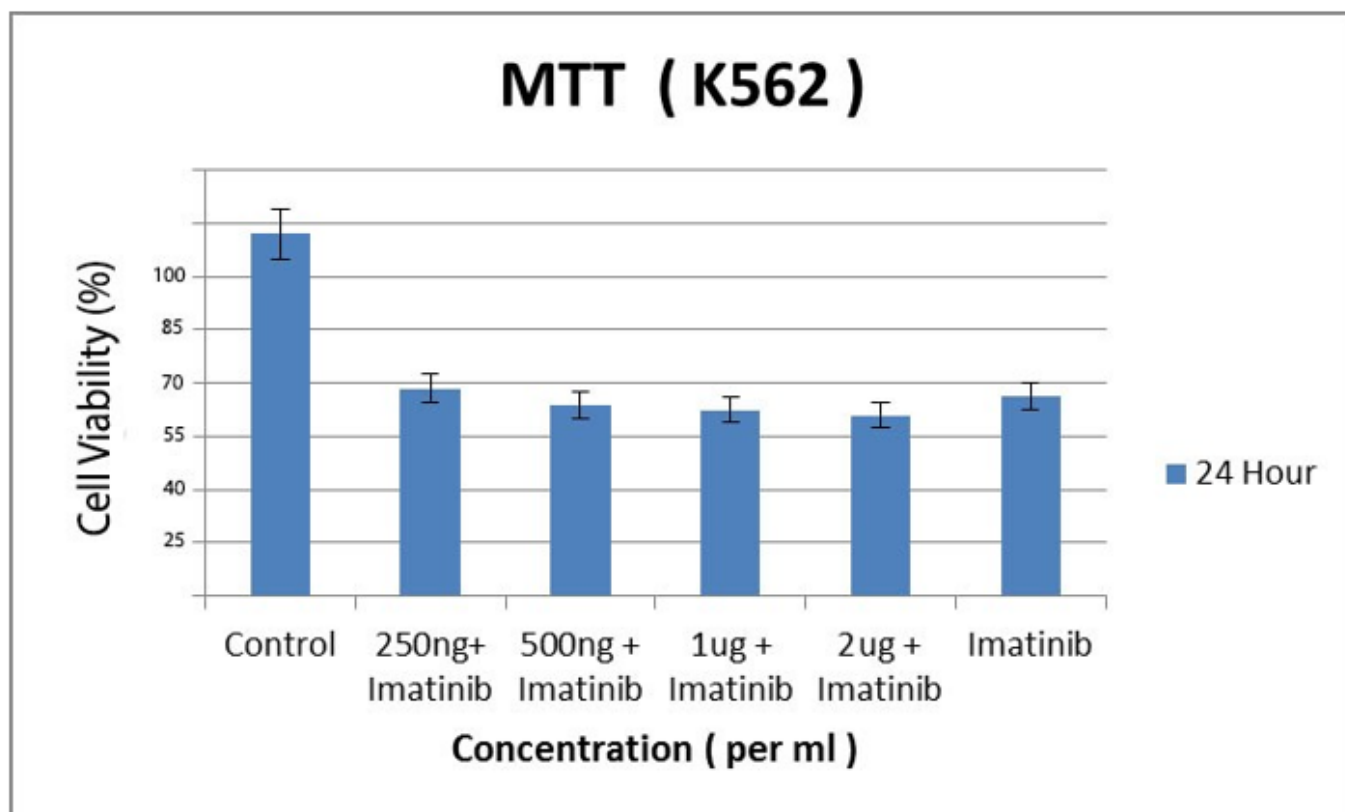


Figure 3

The combined effect of imatinib and Daphne extract on the K-562 cell line was evaluated 24 hr after treatment.

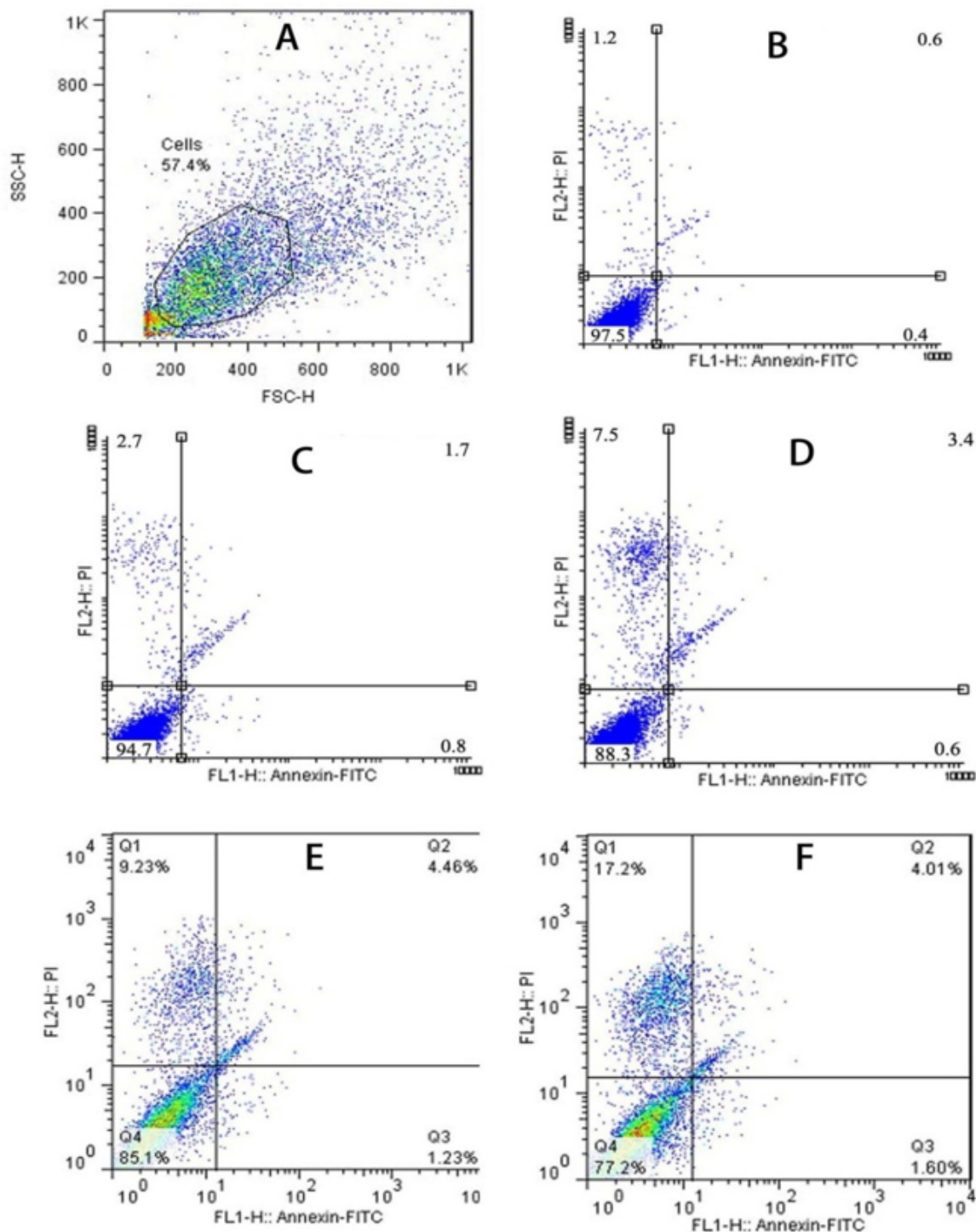


Figure 4

Effects of treatment of K-562 cells with different concentrations of Daphne extract after 24 hr. Gating of the cell population (A). K-562 cells without treatment—normal control (B). Treatment with 125 ng/ml Daphne extract (C). Treatment with 256 ng/ml Daphne extract (D). Treatment with 500 µg/ml Daphne extract (E). Treatment with 1 µg/ml Daphne extract (F).

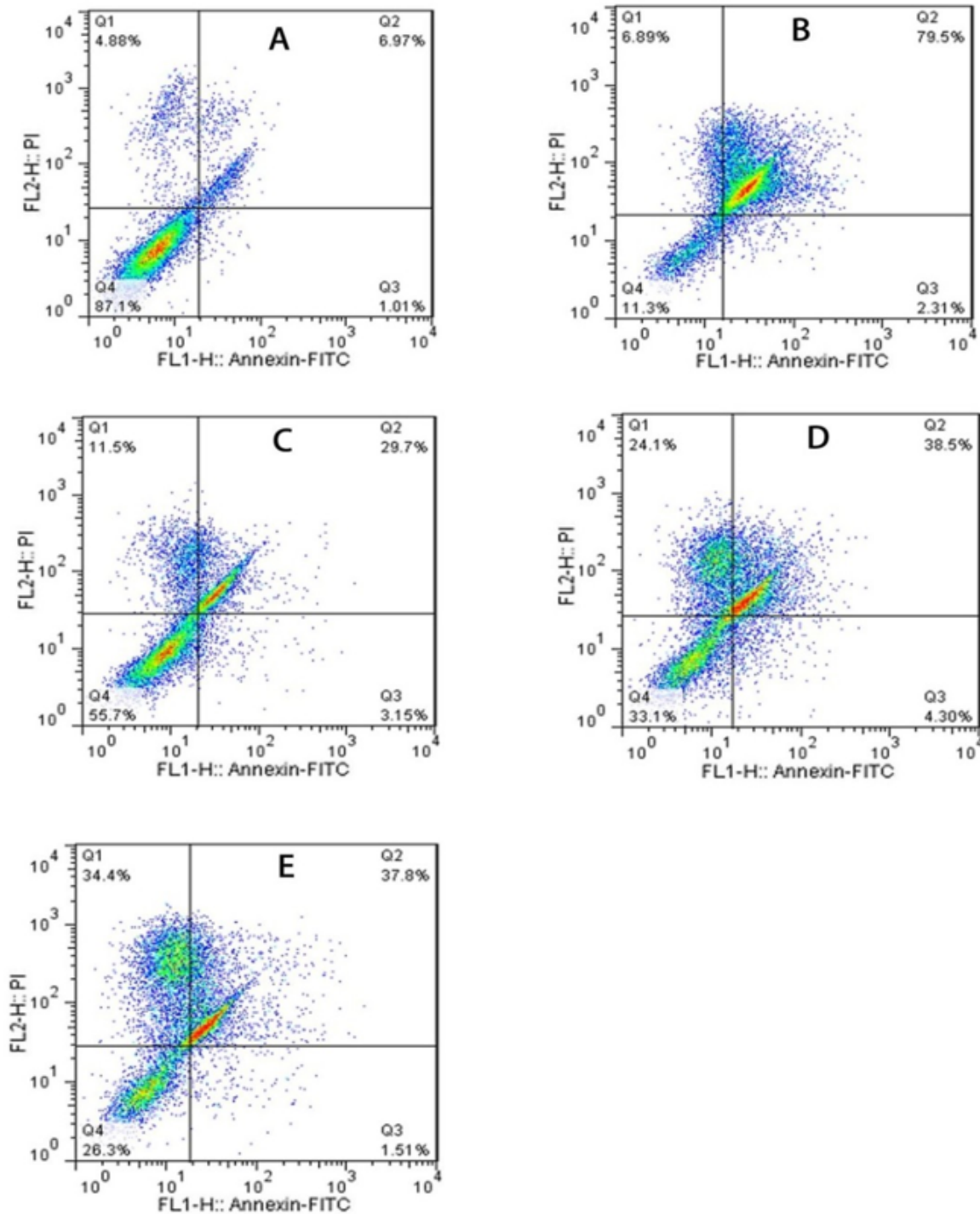


Figure 5

1 μ M Imatinib was combined with different concentrations of Daphne extract on the K562 cell line after 24 h. A control population of cell lines (A). Treatment with imatinib (1 μ M) (B). Combination of imatinib with 500 ng/ml Daphne extract (C). Combination of imatinib with 1 μ g/ml Daphne extract (D). Combination of imatinib with 3 μ g/ml Daphne extract (E).

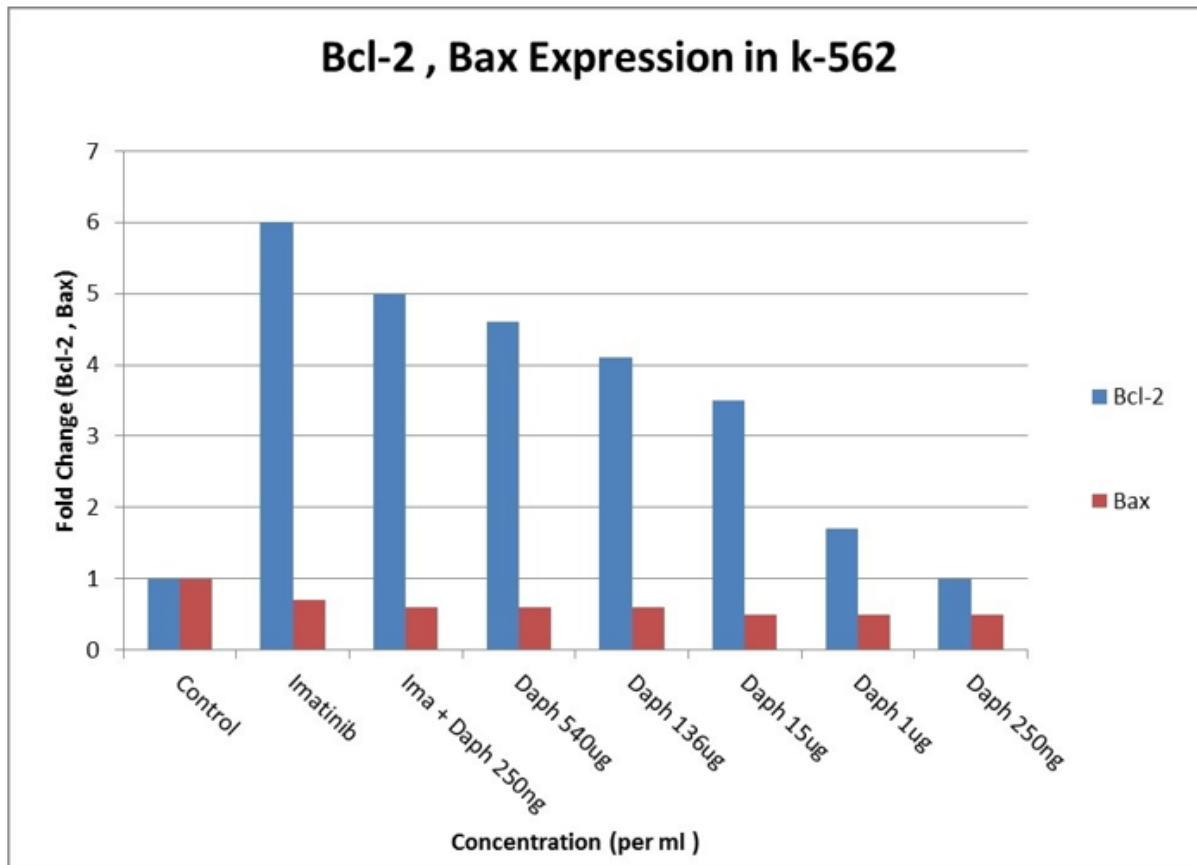


Figure 6

Changes in the expression of Bax and Bcl-2 were detected after treatment with imatinib and Daphne Mucronata.

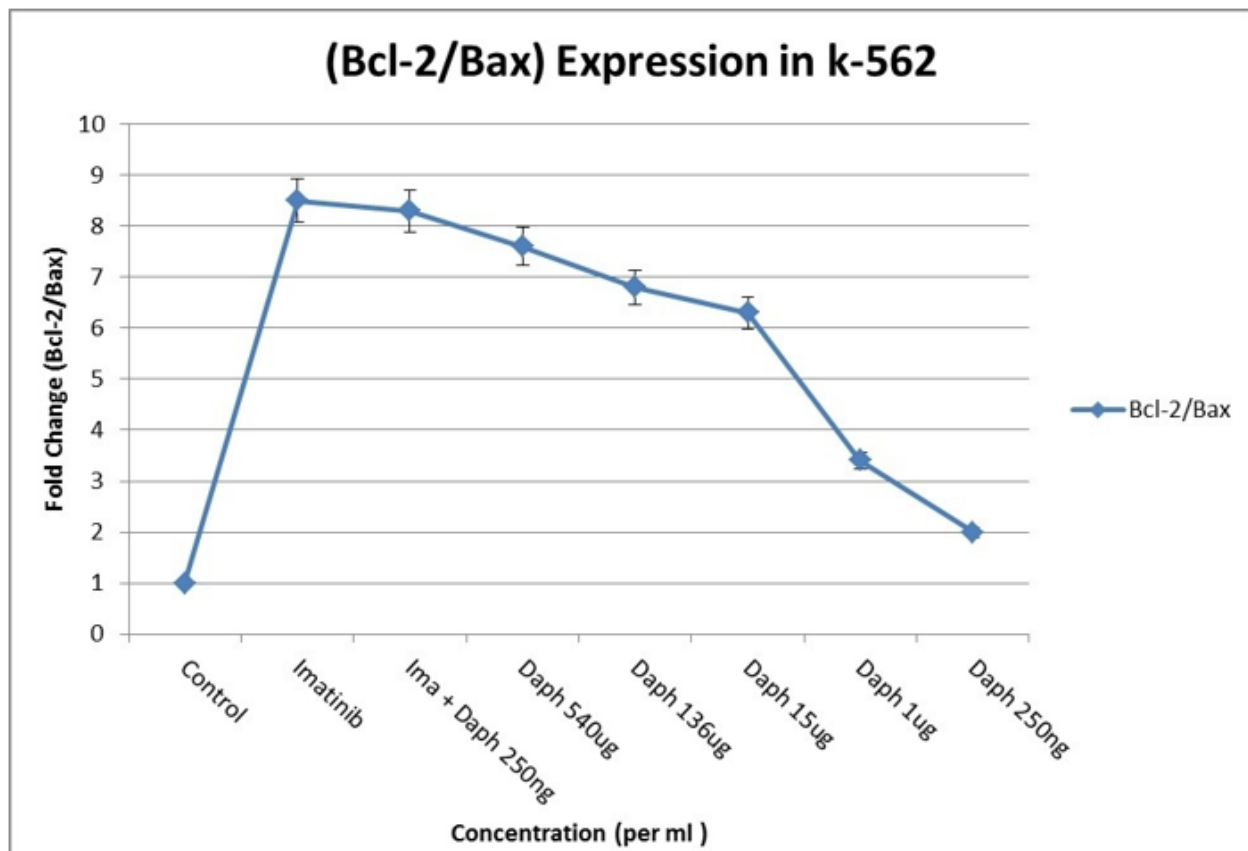


Figure 7

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