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Formononetin and Dihydroartemisinin Act Synergistically to Induce Apoptosis in Human Acute Myeloid Leukemia Cell Lines

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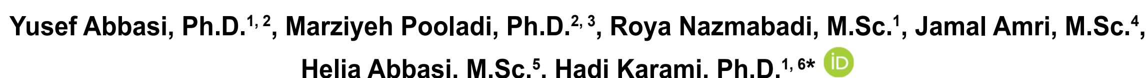

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Abstract

Objective: Enhanced cell survival and drug resistance in tumor cells have been linked to the overexpression of anti-apoptotic members of the Bcl-2 family proteins, including Bcl-2 and Mcl-1. The aim of this study was to explore the impact of formononetin and dihydroartemisinin combination on the growth and apoptosis of acute myeloid leukemia (AML) cells.

Materials and Methods: In this experimental study, the cell survival and cell proliferation were tested by MTT assay and trypan blue staining. The evaluation of cell apoptosis was conducted using Hoechst 33342 staining and a colorimetric assay to measure caspase-3 activity. To determine the mRNA levels of *Mcl-1, Bcl-2, Bax*, and *Cyclin D1*, a quantitative real-time polymerase chain reaction (qRT-PCR) was performed.

Results: We showed that treatment with either formononetin or dihydroartemisinin alone, led to significant decrease in the cell survival and growth, and triggered apoptosis in U937 and KG-1 AML cell lines. Moreover, treatment with each of the compounds alone significantly decreased the mRNA levels of *Mcl-1, Bcl-2 and Cyclin D1* mRNA, while, the expression level of *Bax* mRNA was enhanced. Combination of two compounds showed a synergistic anti-cancer effect.

Conclusion: The anti-leukemic potential of formononetin and dihydroartemisinin is exerted through the effect on cell cycle progression and intrinsic pathway of apoptosis. Therefore, they can be considered as a potential anti-leukemic agent alone or along with existing chemotherapeutic drugs.

Keywords: Acute Myeloid Leukemia, Apoptosis, Bcl-2, Dihydroartemisinin, Formononetin, Mcl-1

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Introduction

Acute myeloid leukemia (AML) is a cancer that specifically affects the myeloid lineage of blood cells, characterized by the malignant transformation of hematopoietic stem/progenitor cells. Despite the optimization of cytotoxic chemotherapy, the complete remission rate is only 30-40%, and recurrence rate is still high in the majority of patients. The main obstacle in treatment of AML patients is the development of drug resistance. Therefore, development of novel therapeutic approached are required for complete cure of AML (1, 2).

The regulation of apoptosis hinges on the interplay between two types of pro-apoptotic and anti-apoptotic proteins and the interaction of these two groups of determines cell death or cell survival (3). Escape from apoptosis is a hallmark of cancer and is important for tumor maintenance and drug resistance. Increased expression of Bcl-2 family anti-apoptotic members such as Bcl-2, Mcl-1 and Bcl-xL has been observed in various malignancies such as AML, which is linked to the enhanced cell survival, decreased apoptosis and enhanced drug resistance (3, 4).

Formononetin is a non-steroidal isoflavonoid that is obtained from various plants such as Astragalus membranaceus and red clover and shows anti-cancer, antioxidant and anti-inflammatory properties (5). Studies have shown that formononetin inhibits growth, migration and cell cycle and induces apoptosis in breast, colorectal, prostate and lung cancers (5, 6). Also, other studies have shown that formononetin changes the expression of *Bcl-2*, *Bax*, *P21*, and *P53* genes, activates caspases, and induces cell death by inhibiting PI3K, NF-kB, JAK/STAT, and MAPK signaling pathways. However, the precise molecular mechanisms underlying the impact of formononetin on AML cells remain elusive (5-8).

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Artemisinin is an active ingredient of the Artemisia annua plant, which is used worldwide as an anti-malarial drug. Dihydroartemisinin is one of the most powerful artemisinin derivatives that have shown various anticancer effects (9). Studies have indicated that this compound suppresses the growth and invasion of cancer cells by mediating oxidative stress, apoptosis, cell cycle, angiogenesis and DNA repair systems. On the other hand, dihydroartemisinin changes the expression of *Bax*, *P21*, *Bcl-2* and *Mcl-1* genes in different tumor cells by inhibiting various signaling pathways such as β-catenin and MAPK (9-14).

Today, combination therapy is proposed as one of the potential and attractive strategies for cancer treatment. In this study, for the first time, the effect of the combination of formononetin and dihydroartemisinin on the growth and apoptosis of AML cancer cells has been investigated.

Materials and Methods

Cell culture

In this experimental study, the U937 and KG-1 AML cell lines were used (Pasteur Institute, Tehran, Iran). All cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) that was containing 20% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 IU/ml penicillin (Sigma-Aldrich, USA) and 100 mg/ml streptomycin (Sigma-Aldrich, USA). Cells were used in the third passage.

Cytotoxicity assay

The cytotoxic effects of formononetin ($C_{16}H_{12}O_4$) and dihydroartemisinin ($C_{15}H_{24}O_5$), both obtained from Cayman (Ann Arbor, MI, USA), were evaluated on AML cells using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. The purpose of this assay was to determine the potential cytotoxicity of these compounds when used alone or in combination. The assay was divided into five groups: blank control, solvent control, formononetin (0-80 μM), dihydroartemisinin (0-80 μM) and formononetin+dihydroartemisinin. All cell lines treated with only 1% DMSO, solvent of formononetin and dihydroartemisinin, were served as solvent control. In brief, leukemic cells were plated in 96-well at a density of 2×104 cells/well. All cells were incubated for 24 hours. Then, the cells were exposed to formononetin and dihydroartemisinin with different concentration and continued to further incubate for 24 hours. Firstly, the cultures were supplemented with 10 µl of MTT solution (Sigma-Aldrich, USA) at a concentration of 5 mg/ml. Following this addition, the cultures were incubated for a duration of 4 hours at a temperature of 37°C. Subsequently, the plate containing the cultures was subjected to centrifugation, resulting in the separation of the supernatants. The supernatants were then discarded, and the cells were treated with 150 µl of DMSO. To determine the absorbance at 490 nm, an ELISA plate reader from Awareness Technology located in Palm City, FL, USA was employed. This measurement was crucial in assessing the survival rate (SR) of the cells. The SR was calculated using the equation: SR (%)=(A Treatment/A Control)×100%. Here, a treatment represents the absorbance of the treated cells, while A Control corresponds to the absorbance of the control cells. By utilizing this equation, the SR of the cells could be quantified accurately. The concentration that reduced 50% of cell survival (IC_{50}) was determined by GraphPad software (GraphPad Software Inc., San Diego, CA, USA). In the next experiments the IC_{50} doses of compounds were used.

Combined effect test

The effect of formononetin and dihydroartemisinin combination therapy on cell survival was performed with the combination index (CI) analysis based upon the Chou-Talalay method (15, 16). The data of MTT assay were converted to fraction affected (Fa, range 0-1; where Fa=0 represents 100% cell survival and Fa=1 represents 0% cell survival) and assessed using CompuSyn 1.0 software from Combosyn (Paramus, NJ, USA). A CI value equal to 1 indicates additive effects, less than 1 indicates synergistic and greater than 1 indicates antagonistic interaction.

Cell viability assay

The antiproliferative effects of formononetin and dihydroartemisinin were determined by trypan blue staining. In brief, 5×10^4 leukemic cells were treated with formononetin and dihydroartemisinin alone and in combination; in six well cell culture plates and then incubated for 24-120 hours. The tumor cells were gathered at various time intervals and subjected to staining with a 0.4% solution of trypan blue (Merck KGaA, Darmstadt, Germany). Following duration of 3 minutes, the count of living cells was ascertained using a hematocytometer and an inverted microscope (Nikon Instrument Inc., Melville, NY, USA). The percentage of viable cells for the blank control group was considered as 100%.

Quantitative real time polymerase chain reaction

Following treatments, total RNA was isolated from the U937 and KG- cells using RNA extraction reagent (Parstous, Iran) according to the manufacturer's instructions. Then, 1 µg of total cellular RNA was reverse transcribed into cDNA by use of MMLV reverse transcriptase, random hexamers and oligi-dT according to the manufacturer's protocol (Parstous, Iran). Relative gene expression was quantified by RT-qPCR using SYBR Premix Ex Taq kit (Parstous, Iran) and the LightCycler 96 system (Roche Diagnostic GmbH, Mannhein, Germany). Each PCR was performed in a 20 µl reaction system containing: 0.2 µM of each of the primers, 12 µl of Master Mix reagent, 1 µl of cDNA template and 6 µl of nuclease-free distilled water. The sequences of primers used for PCR are listed in Table 1. The PCR condition was 95°C for 10 min followed by 35 cycles at 95°C for 20 seconds and 60°C for 1 minute. Relative quantitation was measured with the $2^{-(\Delta\Delta Ct)}$ method (17, 18), using *β-actin* as housekeeping gene.

Table 1: Primer sequences used in real-time polymerase chain reaction

Genes	Primer sequence (5´-3´)	Primer length (base)	Tm (°C)
β-actin	F: GACATCCGCAAAGACCTGTA	20	57.62
	R: GGAGCAATGATCTTGATCTTCA	22	56.22
Bcl-2	F: GGATGCCTTTGTGGAACTG	19	56.50
	R: CAGCCAGGAGAAATCAAACAG	21	57.16
Bax	F: GCTTCAGGGTTTCATCCAG	19	55.88
	R: TTACTGTCCAGTTCGTCCC	19	56.39
Cyclin D	F: GCTCTCGGGTGCTGTATTG	19	58.32
	R: CAATGACCCCGCACGATTTC	20	59.90
Mcl-1	F: TAGTTAAACAAAGAGGCTGGGA	22	57.01
	R: CCTTCTAGGTCCTCTACATGG	21	56.33

Hoechst staining for apoptotic cells

After 24 hours of treatment with IC_{50} concentrations of formononetin and dihydroartemisinin, the cells were washed with phosphate buffer saline (PBS, Invitrogen, Carlsbad, CA, USA) and fixed in 4% formaldehyde (for 15 minutes) at room temperature. Subsequently, the cells were washed with PBS and stained with 5 µg⁄mL Hoechst 33342 (Sigma-Aldrich, USA) for 10 minutes. The nuclear morphological changes were observed under inverted fluorescent microscope (Olympus, Japan).

Caspase-3 activity assay

The *in vitro* activity of caspase-3 was quantified by a colorimetric caspase activity Kit (Abnova, Taiwan) (19). Briefly, the treated cells were lysed in 50 µl of lysis buffer and then centrifuged in 12,000 g for 3 minutes. Next, 5 µl of the DEVD-pNA substrate and 50 µl of reaction buffer were added to each sample. After 2 hours of incubation at 37°C, the absorbance was measured at 405 nm using a microplate reader (Awareness Technology, Palm City, FL, USA).

Statistical analysis

Quantitative data were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Tukey test was used to determine the significant differences among groups. Values of $P<0.05$ were considered significant. GraphPad Prism software version 5 (GraphPad Software Inc., San Diego, CA, USA) was employed to carry out the statistical analyses in this study.

Ethical approval

This research was ethically wise approved from Deputy of research and technology, Arak University of Medical Sciences, Arak, Iran (IR.ARAKMU.REC.1401.176).

Results

Formononetin and dihydroartemisinin inhibited the cell survival of AML cells

To investigate the cytotoxic effect of formononetin and dihydroartemisinin on AML cells, a combination treatment of formononetin and dihydroartemisinin on U937 and KG-1 cells was studied. The MTT assay data showed that treatment whit either formononetin or dihydroartemisinin alone, significantly reduced the cell survival in a dose-dependent way (Fig.1, relative to the blank control). As shown in Table 2, the IC_{50} values for formononetin and dihydroartemisinin for 24 hours of treatment were 31.71 and 13.92 µM in U937 cells, and 46.59 and 11.26 µM in KG-1 cells, respectively. Moreover, combination treatment with formononetin and dihydroartemisinin further reduced the cell SR and lowered the IC_{50} ($P<0.05$, relative to the single therapy).

Table 2: IC_{50} values of the formononetin and dihydroartemisinin, alone and in combination, in U937 and KG-1 AML cell lines

Compound	IC_{50} (24 hours)	
	U937	KG-1
Formononetin	31.71	46.59
Dihydroartemisinin	13.92	11.26
Combination	8.24*	6.68*

IC_{50}; Half-maximal inhibitory concentration, AML; Acute myeloid leukemia, and *; P<0.05 versus single treatment.

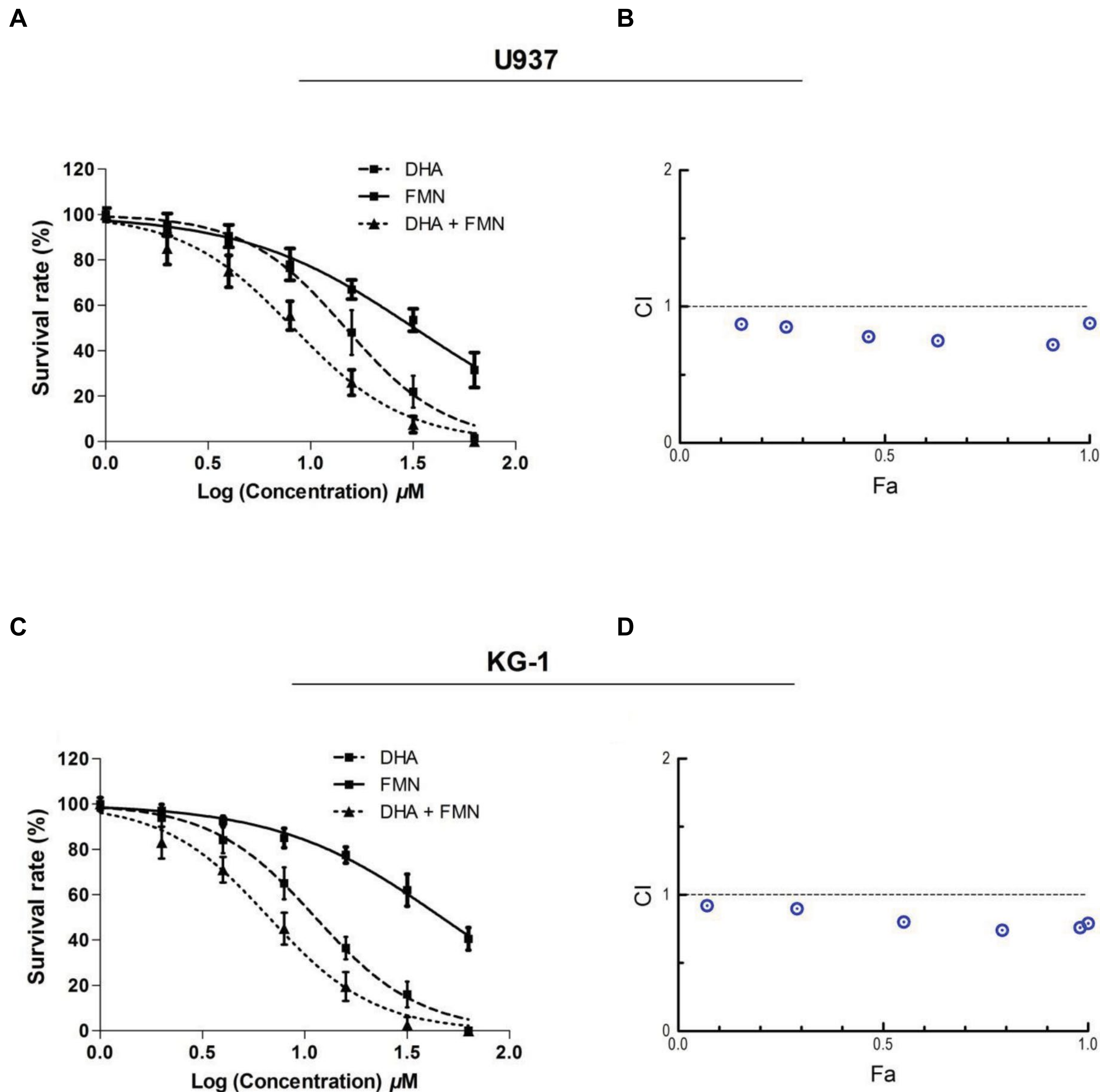

Fig.1: Effect of formononetin (FMN) in combination with dihydroartemisinin (DHA) on cell survival. The U937 and KG-1 AML cells were treated with FMN and DHA at indicated concentrations. The MTT assay was employed to evaluate cell viability twenty-four hours post-treatment. GraphPad Prism was utilized to generate the cell survival curves. The data presented in this study are expressed as the mean ± SD of three experiments. Utilizing the fractional affected (Fa) values derived from the MTT assay, the combination index (CI) values were computed using the CalcuSyn software. **A, B.** Show the cell survival and CI plots for U937-MG cells. **C, D.** Show the cell survival and CI plots for KG1 cells.

The synergistic impact of formononetin and dihydroartemisinin on AML cells was observed through their combined effect

To assess whether the combination effect of formononetin and dihydroartemisinin on cell survival is synergistic effect, we performed the CI analysis using CompuSyn software. The results showed that the combination effect of formononetin (0-64 nM) with dihydroartemisinin (0-64 μM) on AML cells was synergistic (CI<1) in all of concentrations. Our results also indicated that the best synergistic effect of 24 hours was obtained at 32 μM dihydroartemisinin in combination with 32 μM formononetin (CI=0.72), with Fa level of 0.91 in U937 cells (Fig.1). Also, in KG-1 cells the best synergistic effect was obtained at 16 μM dihydroartemisinin in combination with 16 μM formononetin (CI=0.74), with Fa level of 0.79 (Fig.1).

Dihydroartemisinin augmented the growth inhibitory effect of formononetin

We next investigated whether formononetin and dihydroartemisinin could control the growth of AML cells. The tumor cells were exposed to the formononetin and dihydroartemisinin, alone and in combination. By utilizing the trypan blue dye exclusion assay, the measurement of cell viability was performed for a total of 120 hours. The data are expressed as the percentage of viable cells compared to the total number of cells. The

cell growth curve showed that the viability of the cells in formononetin, dihydroartemisinin and combinational group significantly decreased time-dependently, compared with the blank control group. Moreover, at 24 h after treatment, the cell growth of U373-MG cells in formononetin, dihydroartemisinin and combinational groups dropped to 82.5, 73.3 and 70.4%, then to a further 28.3, 19.8 and 16.2%, respectively, at the end of the assay (P<0.05, Fig.2). The KG-1 cell line exhibited comparable outcomes in the study.

Formononetin and dihydroartemisinin changed the expression of cell cycle and apoptotic genes

qRT-PCR method was used to investigate the 24 hours effect of formononetin and dihydroartemisinin on mRNA expression in AML cells. The results demonstrated that single tharapy with each of formononetin or dihydroartemisinin significantly reduced the *Mcl-1, Bcl-2* and *Cyclin D1* mRNA levels, while the expression of *Bax* mRNA increased compared to the control group (Fig.3). In the combination treatment group, stronger effects were observed relative to the single treatment (P<0.05). Additionally, no substantial variations in mRNA expression were observed when comparing the blank control group with the solvent control group (P<0.05). The PCR results were consistent across both U937 and KG1 cell lines (Fig.3).

Formononetin and dihydroartemisinin triggered apoptosis in AML cells

To determine whether the cytotoxic effect of treatments on AML cells was related to the induction of apoptosis, the U937 and KG-1 cells were exposed to the IC_{50} doses of dihydroartemisinin, formononetin) and their combination for 24 hours. Then, Hoechst 33342 staining and caspase-3 activity assay we performed. As shown in Figure 4, apoptotic cells containing nuclear morphologic changes were observed in dihydroartemisinin and formononetin groups but not in the control groups. Moreover, the number of apoptotic cells in the combination group was not significantly differing from the single treated group.

In order to explore the underlying molecular process of apoptosis triggered by dihydroartemisinin and formononetin, a caspase-3 activity assay was performed. The findings from this study revealed a significant elevation in caspase-3 activity when cells were treated with either dihydroartemisinin or formononetin, compared to the control cells (P<0.05). Interestingly, when the AML cells were exposed to a combination of two compounds, there was no significant difference in caspase-3 activity compared to cells treated with either compound alone (P>0.05, Fig.4).

The IC_{50} dose of the combination is lower than the IC_{50} dose of either compound alone; therefore, we conclude that the combination treatment has a stronger effect on triggering apoptosis compared to the single treatment.

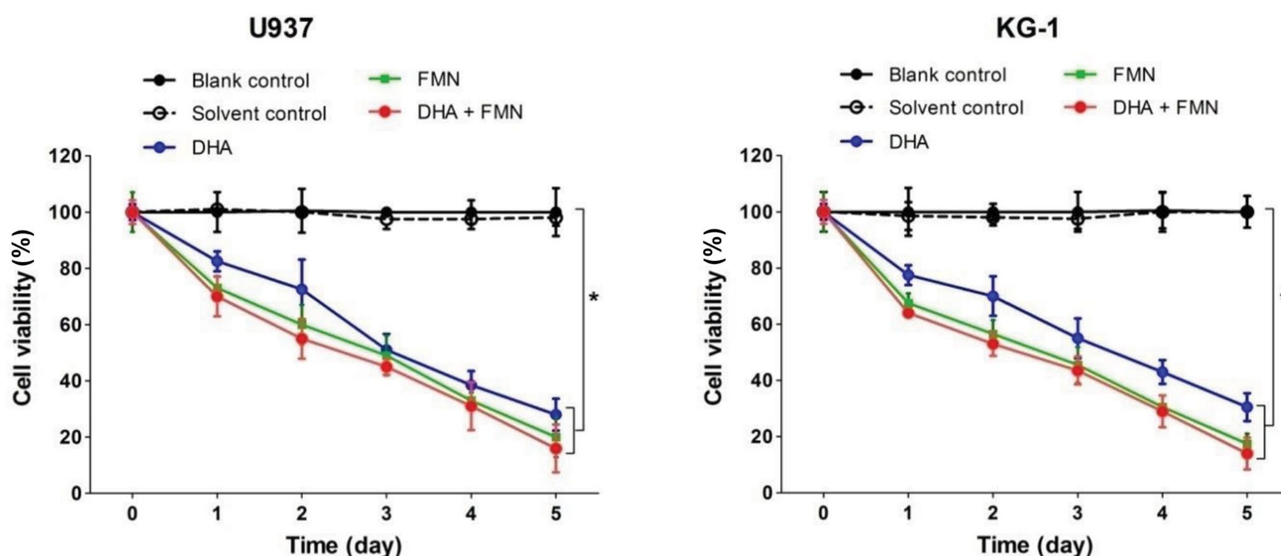

Fig.2: Growth curve of AML cells treated with formononetin (FMN)and dihydroartemisinin (DHA). The U937 and KG-1 cells were treated with FMN and DHA. Next, the cell viability was determined using trypan blue exclusion assay during a period of 5 days. The data is presented as the mean ± SD of three separate experiments. *; P<0.05 vs. blank control or solvent control and AML; Acute myeloid leukemia.

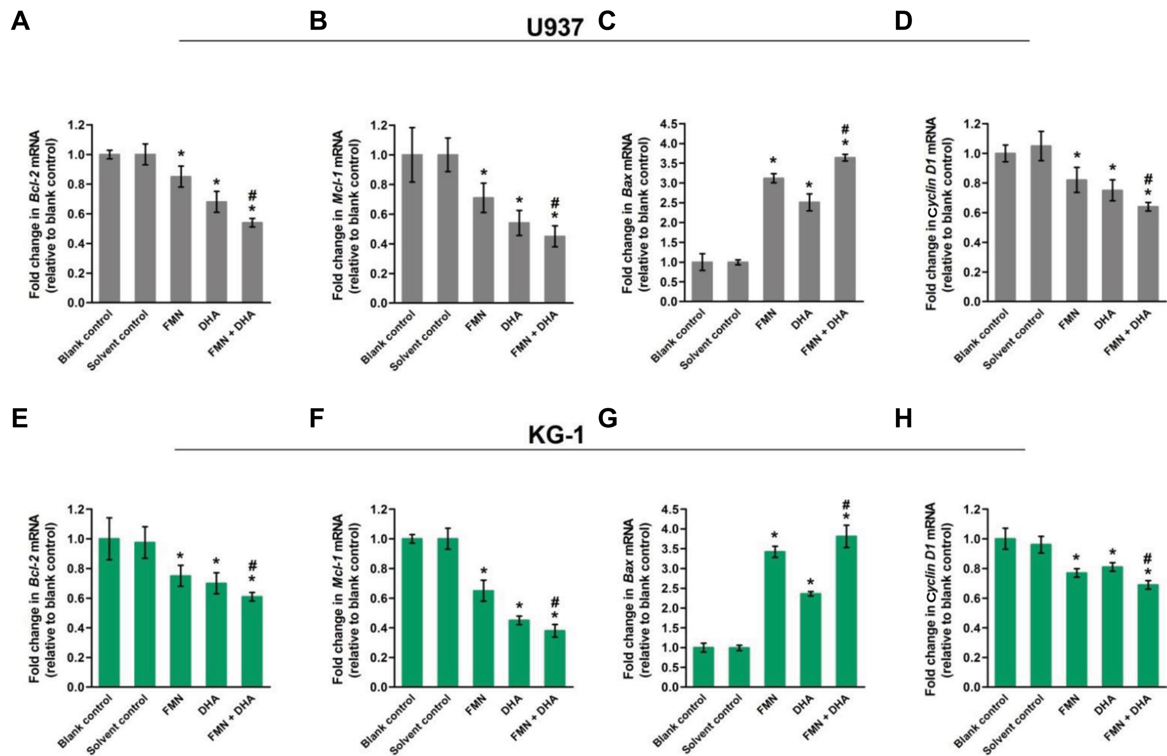

Fig.3: RT-qPCR analysis of leukemic cells. The U937 and KG-1 AML cells were exposed to dihydroartemisinin (DHA) and formononetin (FMN) (IC_{50} doses) for 24 hours. Then, relative mRNA expression levels of **A, E.** *Bcl-2*, **B, F.** *Mcl-1*, **C, G.** *Bax* and **D, H.** *Cyclin D1* were calculated using RT-qPCR and $2^{-(\Delta\Delta Ct)}$ method. Results are expressed as mean ± SD (n=3). #; P<0.05 relative to monotherapy, *; P<0.05 relative to blank control, RT-qPCR; Reverse transcription quantitative polymerase chain reaction, and AML; Acute myeloid leukemia.

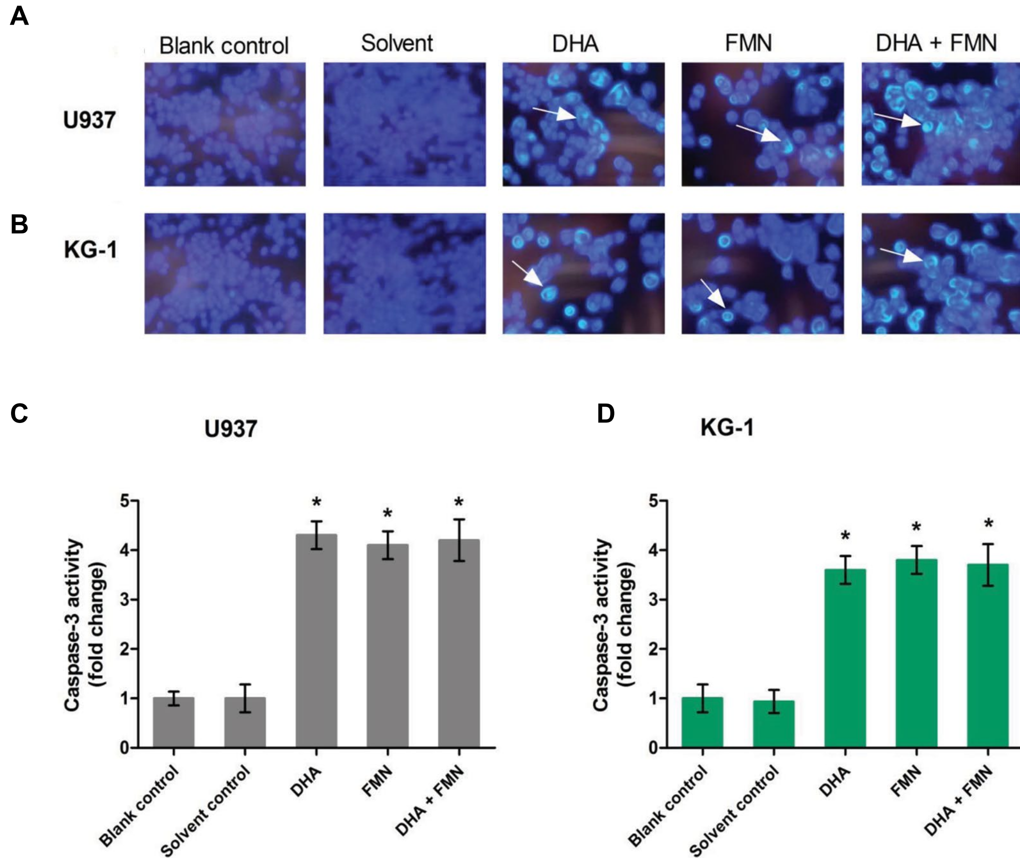

Fig.4: The effect of dihydroartemisinin (DHA) and formononetin (FMN) on apoptosis of AML cells. U937 and KG-1 AML cells were exposed to the IC_{50} doses of DHA and FMN, alone and in combination. Following 24 hours, **A, B**. The apoptosis was assessed using Hoechst 33342 staining and **C, D.** Caspase-3 activity assay. Arrows show the apoptotic cells. The results are presented as mean ± SD (n=3) of three experiments. *; P<0.05 compared with blank control and AML; Acute myeloid leukemia.

Discussion

Despite initial sensitivity to standard chemotherapy, the complete remission rate is only 30-40% in AML, and the majority of patients eventually relapse. As a result, it is of utmost importance to expedite the progress of novel therapeutic interventions that can offer a definitive cure for AML, thereby meeting the urgent demand for effective treatment options (1, 2). Increased expression of Bcl-2 family anti-apoptotic members has been observed in various malignancies such as AML, which is associated with enhanced cell survival, decreased apoptosis and enhanced drug resistance (3, 4). Studies have shown that both dihydroartemisinin and formononetin inhibit growth, migration, cell cycle, and induces apoptosis in cancer cells by altering gene expression as well as inhibiting cellular signaling pathways (5, 6, 9, 11, 20). Combination therapy is now proposed as one of the potential and attractive strategies for cancer treatment. In this study, the effect of the combination of formononetin and dihydroartemisinin on the growth and apoptosis of AML cancer cells has been investigated.

Here we showed that treatment with either formononetin or dihydroartemisinin alone, lead to significant decrease in the cell survival and growth, and triggered apoptosis in U937 and KG-1 AML cell lines. Combination therapy lowered the IC_{50} value and further decreased the cell survival relative to dihydroartemisinin or formononetin alone. The IC_{50} value in combination treatment was lower than the IC_{50} value in either compound alone; therefore, our findings propose that the combination treatment with two compounds has a greater anti-tumor effect relative to the monotreatment. The impact of formononetin and dihydroartemisinin on cancer cells has been extensively examined in various scientific investigations Gao et al. (21) found that dihydroartemisinin triggers apoptosis in human leukemia cells through a process that involves MEK/ERK inactivation, cytochrome c release and caspase activation. Handrick et al. (22) showed that exposure of the Jurkat T-lymphoma cells with dihydroartemisinin causes release of cytochrome c from mitochondria, activation of caspases, and activation of apoptosis.

Furthermore, dihydroartemisinin also enhanced the cytotoxic effect of radiation in lymphoma cells. In another study Zhang et al. (23) explored the combination effect of formononetin and temozolomide on C6 glioma cells. The researchers observed a synergistic impact on C6 cells when temozolomide and formononetin were used in combination, as indicated in their findings. Another research investigated the effect of formononetin on pharyngeal squamous cell carcinoma cells. Results show that formononetin significantly increased squamous cell carcinoma cells cell death; however, it did not affect the viability of normal L929 mouse fibroblasts cells. Tumor cells subjected to formononetin treatment exhibited enhanced features commonly associated with apoptosis, including changes in cell structure, condensation of chromatin, fragmentation of DNA, and an increase in the population of apoptotic cells (24). In this study, we demonstrated that both formononetin and dihydroartemisinin inhibits the cell growth and survival, and triggers apoptosis in U937 and KG-1 AML cells. Moreover, the combination of two agents showed a stronger anti-cancer effect. Our results are in agreement with the above reports and show that both formononetin and dihydroartemisinin can inhibit the growth and proliferation of cancer tumor cells such as AML, and the effect of their combination are synergistic.

We next explored the effect of two compounds on mRNA expression. The qPCR data revealed that treatment with each of the compounds alone significantly suppressed the mRNA levels of *Mcl-1*, *Bcl-2* and *Cyclin D1*, while, the expression level of *Bax* mRNA was enhanced. The effect of the combination of two agents on gene expression was stronger than their individual effect. Due to the relationship between gene expression changes in tumor cells and tumor characteristics such as drug resistance, several studies have been conducted to explore the effect of these compounds on gene expression and sensitization of tumor cells. For example, Yang et al. (6) demonstrated that formononetin suppressed the cell growth, augments cell cycle arrest and enhances cell death in of NCI-H23 and A549 lung cancer cell lines. On the molecular level, they revealed that formononetin treatment inhibits the expression level of *Bcl-2*. In addition, the expression of cell cycle arrest-associated proteins such as cyclin A, P21 and cyclin D1 were decreased after treatment with formononetin. Xin et al. (25) in a study showed that down-regulation of *Bcl-2* mRNA expression by formononetin was associated with the enhancement of apoptosis in MCF-7 breast cancer cells. Moreover, growth inhibitory effect of metformin enhanced after formononetin treatment. Hou et al. (26) in another study demonstrated that dihydroartemisinin enhances cytotoxicity in hematoma cells liver cells. Additionally, this compound exhibited the ability to inhibit the growth of cells, leading to a halt in the progression of the cell cycle, and initiating programmed cell death, known as apoptosis. Moreover, this compound suppressed cell proliferation, induced cell cycle arrest, and triggered apoptosis. Furthermore, therapeutic efficacy of chemotherapeutic agent gemcitabine was enhanced by dihydroartemisinin. Another study on HeLa and HepG2 cells revealed that resveratrol and artemisinin synergistically inhibit the cancer cell growth and migration, and combination of them exhibits greater anti-tumor activity (27). The results of above reports are in agreement with our findings and show that both formononetin and dihydroartemisinin suppress the tumor cell growth and trigger apoptosis by changing the expression of *Mcl-1*, *Cyclin D1*, *Bcl-2* and *Bax* mRNA. Moreover, the combination of two agents shows a synergistic effect which is associated with the reduction of IC_{50} and the effective dose of each compound.

The regulation of the intrinsic apoptosis pathway is dependent on the presence of pro- or anti-apoptotic members within the Bcl-2 family proteins. Under conditions that induce apoptosis, proteins like Bax and

Bak, which possess pro-apoptotic properties, initiate the process by forming oligomers on the outer membrane of mitochondria. This action leads to the permeability of the mitochondrial outer membrane, known as mitochondrial outer membrane permeability (MOMP). Consequently, cytochrome c is released from the mitochondria into the cytoplasm, triggering the activation of caspases. The anti-apoptotic proteins such as Bcl-2 and Mcl-1 prevent apoptosis by inhibiting the activation of pro-apoptotic members on the mitochondrial outer membrane (1, 28, 29). Deregulation of apoptosis is associated with unchecked cancer cell growth and resistance to drug therapies. Several mechanisms for tumor cell resistance to apoptosis induction have been reported, including activation of survival pathways, down-regulation of pro-apoptotic proteins and up-regulation of anti-apoptotic proteins (30). Understanding the mechanisms of apoptosis deregulation in cancer can help develop targeted therapeutic strategies to reactivate apoptosis and overcome drug resistance. Cyclin D1 is also a protein that plays a pivotal role in regulating cell cycle progression and apoptosis. Cyclin D1 has been demonstrated to mediate resistance to apoptosis by up-regulating molecular chaperones and consequent redistribution of cell death regulators. Activation of the pro-apoptotic protein, Bax, is also decreased in cyclin D1-producing cells (31). Moreover, up-regulation of cyclin D1 enhances radiosensitivity and radiation-induced apoptosis in a breast tumor cells (32). Previous studies have shown that both formononetin and dihydroartemisinin exhibit anticancer effects through growth inhibition and apoptosis induction. Through the modulation of cyclin D1, cyclin E, P21, and cyclin-dependent kinases expression, these two compounds induce a halt in the progression of the cell cycle. Furthermore, by decreasing the expression level of Bcl-2, Bcl-xL and Mcl-1 and also increasing the expression of Bax and Bid proteins, they induce apoptosis in cancer cells. These effects of formononetin and dihydroartemisinin on cell cycle and apoptosis are exerted through different cell signaling pathways such as Wnt/β-catenin, PI3K/AKT, ERK/MAPK and JAK/STAT (9, 5). However, the results of our study are in agreement with the above data and indicate the anticancer potential of formononetin and dihydroartemisinin, which is exerted through the effect on cell cycle and apoptosis.

Conclusion

Our study demonstrated that both formononetin and dihydroartemisinin significantly decrease the expression levels of *Mcl-1*, *Bcl-2* and *Cyclin D1* mRNA, while enhance the expression level of Bax mRNA. The anti-leukemic potential of formononetin and dihydroartemisinin is exerted through the effect on cell cycle progression and intrinsic pathway of apoptosis. The combination of formononetin and dihydroartemisinin has a greater effect on AML cell growth, apoptosis and gene expression, relative to monotreatment. In combination therapy, the effect was synergistic and the IC_{50} value was dramatically decreased. Therefore, they can be considered as a potential anti-leukemic agent alone or along with existing chemotherapeutic drugs. The present study was carried out on cell lines. Conducting this study on animal models and human samples will provide better results.

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Authors' Contributions

H.K., Y.A.; Study concept, Design, Critical revision of the manuscript for important intellectual content, and Funding recipients. R.N., M.P., H.A., J.A.; Acquisition of data. H.K., J.A., Y.A., R.N., M.P., H.A.; Analysis and Interpretation of data. Y.A., H.K., H.A., R.N., M.P., J.A.; Drafting of the manuscript. All authors read and approved the final manuscript.

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