**Scholars Journal of Applied Medical Sciences**
Abbreviated Key Title: Sch J App Med Sci
ISSN 2347-954X (Print) | ISSN 2320-6691 (Online)
Journal homepage: https://saspublishers.com

OPEN ACCESS

**Haematology**

# Investigation of Anticancer Effects of Apigenin on MOLT-3 Human Leukaemia Cell Line

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**DOI:** 10.36347/sjams.2022.v10i02.003 | **Received:** 29.12.2021 | **Accepted:** 04.02.2022 | **Published:** 09.02.2022

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**Abstract** **Original Research Article**

Leukaemia is an aggressive blood malignancies characterized by abnormal accumulation and proliferation of WBCs. Among children younger than 15 years, leukaemia consider the most common type of cancer which accounting 30% of all diagnosed malignancies. The Saudi Cancer Registry (SCR) reported that leukaemia ranked as the 5th type of cancer among both male and female. Because of the undesirable side-effects of the chemotherapy commonly used for leukaemia treatment such as high toxicity and development of secondary cancer, the search for novel and new promising treatment with fewer side-effects and higher therapeutic efficiency is still a priority goal. Using bioactive compounds from natural sources was an effective treatment for a variety of blood cancer. Thus, the aim of this study was to use the bioactive compound Apigenin to treat human lymphoid leukaemia cell line MOLT-3 with three different doses (25, 50, 100 µM) for 24 h. The results of this study showed that cellular proliferation was significantly reduced with 25, 50 and 100 µM Apigenin. In addition, significant increase in apoptotic induction was demonstrated when assessed with Annexin V-FITC/ PI and Caspase-3 substrate based on flow cytometry. Cell cycle arrest was significantly reported at S phase within MOLT-3 cells in a dose response manner. Thus, the finding of this study suggested that Apigenin is a potent and efficient anticancer compound through its activity of blocked cell proliferation, apoptotic induction and cell cycle arrest. However, further studies are required to investigate if the dietary intake of Apigenin might interfere with chemotherapeutic treatment of leukaemia.

**Keywords:** Leukaemia, MOLT-3, Apigenin, Cell cycle, Apoptosis.



## INTRODUCTION

Leukaemia is an aggressive and acquired hematopoietic malignancies that characterized by abnormal accumulation and proliferation of the white blood cells (WBCs) and might affected people at any age. However, some of acute myeloid and acute lymphocytic leukaemia are associated with inherited genetic abnormalities, which affecting genes' stability and repair of DNA [1]. Leukaemia is like other type of cancers considers multifactor disorder. On the other hand, a number of possible factors including genetic, environmental or infectious were highly associated with increasing the risk of leukaemia development. Such of these factors are ionizing and nonionizing radiation, chemicals such as pesticides, smoking, alcohol and drug-metabolizing enzymes [2]. Among children younger than 15 years, leukaemia consider the most common type of cancer which accounting 30% of all diagnosed malignancies [3]. According to National Cancer Institute (2018), there were 459,058 leukaemia patients in the United States. Worldwide, the death rate from leukaemia was 311594 among both genders in 2020 [4] . In Saudi Arabia, leukaemia ranked as the 5th type of cancer among both male and female. In addition, leukaemia incidence was higher in males compared to females during the period (1999–2013) [5].

Although, the chemotherapy drugs are one of the best therapeutic option and the most commonly used therapy to treat leukaemia, it had several undesirable side-effects such as resistance to chemotherapeutic medicine, high toxicity, development of secondary cancer and hair loss [6]. Thus, the search for novel and new promising leukaemia treatment with fewer side-effects and with higher therapeutic efficiency is still a

priority goal [7]. Several research studies treated human cancer cell lines such as; lung [8] , breast [9] and gastrointestinal [10] cancers with natural compounds which showed promising anticancer effects through apoptotic induction, cell cycle arrest and inhibition of cellular growth [11].

The accumulated data on treatment of leukaemia by using natural compounds and or extract of natural compounds showed a significant inhibition on cell cycle and cellular proliferation. Inhibition of apoptosis was also reported among several leukemic patients following the treatment with certain natural compounds. Anticaner effects of curcumin was reported to induce apoptosis among chronic lymphocytic leukaemia CLL B-lymphocytes [2], while the healthy cells were less sensitive [12, 13, 14]. Zaini *et al*., reported a significant induction of apoptosis on both human myeloid and lymphoid leukaemia cell lines following 72 h treatment with carrot juice extract when investigated by flow cytometric analysis using annexin V/propidium iodide staining [15]. Another study showed that low-dose of whole pomegranate juice treatments were able to decrease cell viability indicated by reduction in ATP levels of leukemic cells [16]. Within the same study, cell cycle arrest was reported on HL-60 and CCRF_CEM human leukaemia cell lines in dose response manner when treated with pomegranate juice extract for 48 h [16]. Interestingly, the treatment with majority of natural compounds were detected to affect the cancerous cell but not the normal healthy cells. Apigenin belongs to the flavone and found on a variety of vegetables and fruits including; grapes, apples, parsley and chamomile tea [17, 18] . Apigenin is one of the natural compound that highly presented with anti- cancerous effects. Different types of cancers such as melanoma [19], liver cancer [20], colorectal cancer [21], breast cancer [20], lung cancer [22] and prostate cancer [23] were demonstrated with therapeutic response following the treatment with Apigenin. The aim of this study was to investigate the effect of Apigenin on cellular proliferation, apoptotic induction and cell cycle arrest.

## 2. MATERIALS AND METHODS

### *2.1 Cell Line Culture*

Non tumour control CD133 was obtained from Stem Cell Technologies (Grenoble, France) and MOLT-3 human lymphoid leukaemia cell line from patient released following chemotherapy (ATCC: CRL-1552, Middlesex, UK) were used in this study. In 75 cm flask (Invitrogen, Paisley, UK) two million cells were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK) The cells were maintained at 37°C in a humidified incubator 0f 95% air and 5% $CO_2$. The MycoAlert ™ mycoplasma detection kit (Lonza Walkersville, Inc) was used in this study to test all cells for mycoplasma contamination.

### *2.2 Treatment with Apigenin*

Malvidin (Sigma, Poole, UK) was dissolved in ethanol and used to treat leukemia cell lines in addition to non-tumour control cells for 24 h at concentration (0, 25, 50, 100 µM). All treatments in this study were performed in triplicate, in three independent experiments.

### *2.3 Investigating the Inhibition of cellular proliferation Following the Treatment with Apigenin*

*2.3.1 The ATP Level investigation*

In white 96- wells plates (Fisher Scientific, Loughborough, UK), twenty five thousand cells per well of the control cells CD133 as well as MOLT-3 cell line were treated with 0, 25, 50 and 100 µM of Apigenin and incubated for 24 h. Then, 25 µl of cell titer-glo reagent from Cell Titer Glo Luminescent Cell Viability Assay kit (Promega, Southampton, UK) were added to each well, and incubated at room temperature for 10 min. The luminescence was measured using synergy neo2 luminescence detector (BioTek, Oxfordshire, UK).

### *2.4 Investigating Induction of Apoptosis Following the Treatment with Apigenin*

Apoptotic induction was assessed by flow cytometry using Annexin V (BD, Oxford, UK) and propidium iodide (PI) staining (Sigma, Poole, UK). Moreover, induction of apoptosis was confirmed by using caspase 3 activity assay (Cambridge Bioscience, Cambridge, UK) and examined using flow cytometry.

#### *2.4.1 Annexin V/PI Stain analysis using flow Cytometry*

Following 24 h treatment with Apigenin, treated cellular content from each well were moved to Eppendorf tubes and centrifuged for 5 minutes at 400 g, 4 ºC. Then cells were resuspended in 100 µl Dulbecco's Phosphate-Buffered Saline (DPBS) (Invitrogen, Paisley, UK) following removing supernatant. After that, 100 µl binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) (BD, Oxford, UK) was added to wash the cells twice. After that, 50 µl binding buffer and 5 µl of Annexin V were added to cells and incubated in the dark for 20 minutes. Finally, 300 µl of PI (50 µ g / mL) was added and samples directly analysed on the flow cytometer using a BD FACS Calibur flow cytometry and FlowJo software was used for data analysis (Tree Star, Treestar, Ashland, OR, USA).

#### *2.4.2 Caspase 3 Stain analysis using flow Cytometry*

Following 24 h incubation time, 200 µl of cell suspensions from each well were moved to flow cytometry tubes Then, 5 µl of caspase-3 substrate (0.2 mM) (Promega, Southampton, UK) was added to each sample and incubated for 15 minutes in the dark. Finally, a BD FACS Calibur instrument (BD, Oxford, UK) flow cytometry was used to analyse samples and FlowJo software was used for data analysis

### *2.5 Investigating the Cell Cycle Arrest Following the Treatment with Apigenin*

#### *3.5.1 Cell Cycle Analysis*

Following 24 h treatment of MOLT-3 cells with 0, 25, 50 and 100 µM of Apigenin, cells were moved to Eppendorf tubes and centrifuged at 400 g at 4 ºC for 5 minutes. Then, 100 µl cold DPBS was added to wash cells twice following removing supernatant. Following that, 100 µl of 80 % of cold ethanol was added to fix the cells and stored in dark at -20 °C overnight. Then, 300 µl of PI (Sigma, Poole UK) (50 µg/ mL) and 50 µl of RNase (0.1 unit/ mL) (Sigma, Poole UK) were added to cells following washing twice with cold DPBS. After that, Cells were stored at 4 ºC for 24 h. Finally, a BD FACS Calibur instrument (BD, Oxford, UK) flow cytometry was used to analyse samples and FlowJo software was used for data analysis.

### *2.6 Statistical Analysis*

For each assay means and standard error of the mean (SEM) were calculated. SPSS software was used to indicate whether data parametric or no-parametric. As data were non-parametric a Kruskal–Wallis and Conover–Inman post hoc tests were used to investigates statistical significance of the data. Results were considered statistically significant when P⩽0.05.

## 3. RESULTS

### *3.1 Effect of Apigenin on inhibition of cell proliferation*

A significant ($P \leq 0.05$) decrease in the ATP level of MOLT-3 leukaemia cell line culture was reported after 24 h treatment when assessed with Cell Titer-Glo® Luminescent Cell Viability Assay. The greatest cellular inhibition on MOLT-3 cells was seen with 50 and 100 µM treatment with Apigenin with 40% and 28% of cells remaining live respectively. In addition, the IC50 of MOLT-3 cells was detected with 38 µM of Apigenin. However, the normal control cells showed less than 25% decrease on cell proliferation with the highest dose used in this study (Figure 1).

### *3.2 Effect of Apigenin on Apoptosis*

#### *3.2.1 Apoptotic detection by Annexin V-FITC/ PI based on flow cytometry*

The results of this study demonstrated a significant ($P \leq 0.05$) decrease in the number of live cells of MOLT-3 lymphoid leukaemia cell lines when treated with Apigenin at concentrations of 25, 50 and 100 µM (Figure 2). Similarly, a significant ($P \leq 0.05$) increase on apoptotic induction on MOLT-3 following 24 h treatment with 25, 50 and 100 µM of Apigenin in a dose response manner (Figure 2). More than 60% of MOLT-3 were apoptotic and 33% dead with 100 µM Apigenin.

#### *3.2.2 Apoptotic detection by Caspae-3 based on flow cytometry*

Induction of apoptosis was confirmed with assessment of Caspase-3 activation using NucView™ 488 Caspase-3 substrate based on flow cytometry. The result of this study showed significant ($P \leq 0.05$) increase on apoptotic induction on MOLT-3 cells after 24 h treatment with Apigenin. Apoptotic induction was seen with a dose manner and showed 37%, 75% and 93% cells were apoptotic with 25, 50 and 100 µM respectively (Figure 3).

### *3.3 Effect of Apigenin on Cell Cycle*

Treatment of MOLT-3 leukaemia cells lines with the Apigenin for 24 h demonstrated significant ($P \leq 0.05$) S phase arrest as well as significant decrease in cells within G0/G1 phase. However, no significant changes on M phase was observed following Apigenin treatment on MOLT-3 cells for 24 h (Figure 4).

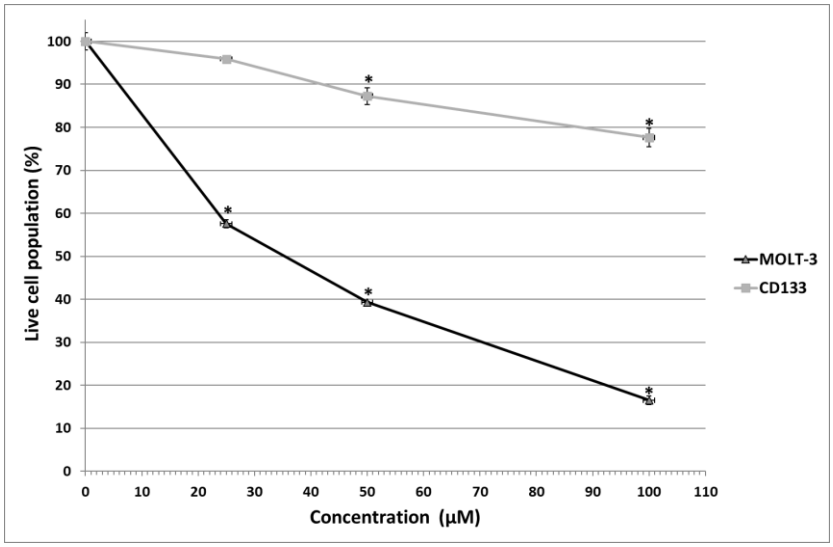

**Figure 1: Cytotoxic effect of Apigenin on inhibition of cell proliferation on leukaemia cell line (MOLT-3) and non-tumor control cell (CD34) using Cell Titer-Glo® Luminescent Cell Viability Assay to indicate live cell number. Cells treated at concentration (0, 25, 50 and 100 µM) for 24 h. ATP level were normalized to control. significant difference (P ≤ 0.05) versus untreated control is indicated by (*)**

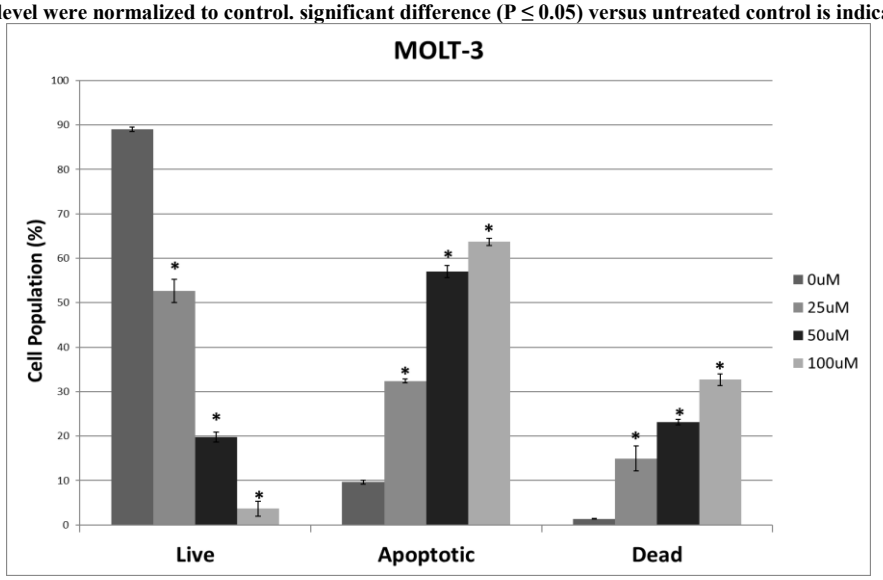


**Figure 2: Effect of Apigenin on induction apoptosis on lymphoid leukemia cell line (MOLT-30) using Annexin V-FITC/ PI based on flow cytometry. Cells treated at concentration (0, 25, 50 and 100 µl) for 24 h. Significant difference (P ≤ 0.05) versus untreated control is indicated by (*)**

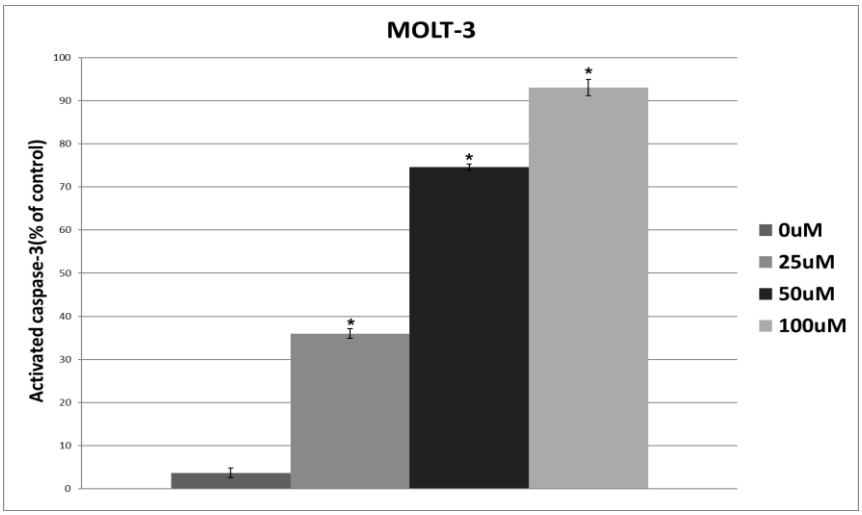


**Figure 3: Effect of Apigenin on caspase-3 activation on myeloid leukemia cell line (MOLT-3), lymphoid leukemia cell line using NucView™ 488 Caspase-3 substrate based on flow cytometry. Cells treated at concentration ( 0, 25, 50 and 100 µM) for 24 h. significant difference (P ≤ 0.05) versus untreated control is indicated by (*). Mean ± SEM**

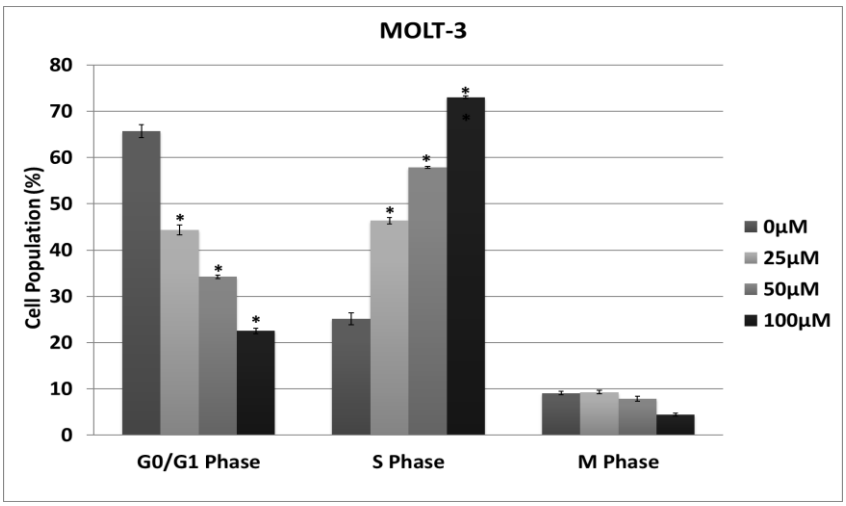

**Figure 4: Effect of Apigenin on cell cycle arrest on lymphoid leukaemia cell line (MOLT-3) using PI satin based on flow cytometry, following treatment with malvidin at concentration (0, 25, 50 and 100 µM) for 24. Significant difference (P ≤ 0.05) versus untreated control is indicated by (*)**

## 4. DISCUSSION

Leukaemia is an aggressive blood malignancies that characterized by abnormal accumulation and proliferation of WBCs. Among children younger than 15 years, leukaemia consider the most common type of cancer which accounting 30% of all diagnosed malignancies [3]. The Saudi Cancer Registry (SCR) reported that leukaemia ranked as the 5th type of cancer among both male and female [5]. Because of the undesirable side-effects of the chemotherapy commonly used for leukaemia treatment such as high toxicity and development of secondary cancer [6], the search for novel and new promising treatment with fewer side-effects and higher therapeutic efficiency is still a priority goal. Using bioactive compounds from natural sources was an effective and promising treatment for a variety of blood cancer [16, 15, 12, 7]. Such natural compounds and chemicals were detected to have an anticancer effects through blocked cellular proliferation, apoptotic induction and cell cycle arrest [24, 25]. In this study, the bioactive compound Apigenin was used to treat human lymphoid leukaemia cell line MOLT-3 with three different doses for 24 h.

The current study demonstrated that Apigenin responsible for inhibition of MOLT-3 cellular proliferation with only 40% and 28% cells remaining live when treated with 50 and 100 µM respectively. In addition, the IC50 of MOLT-3 was observed with 38 µM of Apigenin . Similarly, Mahboub et al (2013) showed significant inhibition on cellular proliferation among different leukaemia cell lines including; HL-60, K562 and U937 when treated with Apigenin for 24 h [26]. Interestingly, the normal healthy cells CD133 were less sensitive to the Apigenin treatment when compared to leukaemia cell lines MOLT-3 cells in this study. Matching with this study's result was reported in several research studies [27, 25, 16, 28].

Induction of apoptosis following treatment with Apigenin on MOLT-3 cells were assessed by using Annexin V-FITC/ PI and Caspase-3 substrate based on flow cytometry. The result from this study showed significant ($P \leq 0.05$) increase in the number of apoptotic cells in dose-responsive manner after 24 h treatment with Apigenin. Triggering cell apoptosis by Apigenin has been reported on HL-60 cells through upregulating of pro-apoptotic proteins [29]. Another study showed a rapid induction of caspase-3 activity within human leukaemia cell line HL-60 when treated with 60 µM of different flavonoids, while the most potent was Apigenin followed by quercetin and myricetin [30].

This study also showed significant ($P \leq 0.05$) and potent inhibitory effect on proliferation of MOLT-3 cells through cell-cycle arrest in S phase. However, arrest within G2/M phase was reported when HL60 cells treated with Apigenin, whereas TF1 cells were arrested at different stage of the cell cycle in the G0/G1 phase [31]. The variation between the result of this study and other research finding used Apigenin treatment in cell cycle arrest at different phases might refer to using different leukaemia cell lines and different doses.

## 5. CONCLUSION

This study showed that Apigenin is a potential chemopreventive agent due to its anticancer activities through leukaemia cellular proliferation inhibition, induction of apoptosis and cell cycle arrest. However, further studies are required to investigate if the dietary intake of Apigenin might interfere with chemotherapeutic treatment of leukaemia.

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