

**THERAPEUTIC POTENTIALS OF FISETIN,
HESPERETIN AND VITEXIN ON CHRONIC
MYELOID LEUKEMIA AND ACUTE MYELOID
LEUKEMIA CELLS**

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ABSTRACT

THERAPEUTIC POTENTIALS OF Fisetin, Hesperetin and Vitexin on Chronic Myeloid Leukemia and Acute Myeloid Leukemia Cells

Fisetin, hesperetin and vitexin are plant-derived flavonoids. This thesis study aims to investigate therapeutic potentials of them on human HL60 APL and K562 CML cells since there are no studies on these cells. The effects of these compounds on APL and CML cells have been considered in terms of cytotoxicity, apoptosis and cell cycle progression. In this study, genome-wide microarray analysis has been also performed for APL and CML cells to identify the genes and networks that are responsible for fisetin and hesperetin-induced effects. In summary, we intended to explain the molecular mechanisms and global gene expression patterns related with the effects of these flavonoids on both APL and CML for the first time.

There were decreases in the viability/proliferation of K562 and HL60 cells treated with fisetin, hesperetin and vitexin. Fisetin was the most effective flavonoid for the induction of apoptosis in both cells. Fisetin, hesperetin and vitexin have been found to affect cell cycle progression at different phases of the cell cycle in both CML and AML cells, thus having cytostatic effects.

In conclusion, the results of this study indicated that especially fisetin and hesperetin may have therapeutic potential in APL and CML cells due to induction of apoptosis, inhibition of cell proliferation and cell cycle arrest. Moreover, the genetic networks derived from this study illuminate some of the biological pathways affected by fisetin and hesperetin treatment while providing a proof of principle for identifying candidate genes that might be targeted for CML and APL therapy.

ÖZET

FİSETİN, HESPERETİN VE VİTEKSİN'İN KRONİK MİYELOİD LÖSEMİ VE AKUT MİYELOİD LÖSEMİ HÜCRELERİ ÜZERİNE TERAPÖTİK POTANSİYELLERİ

Fisetin, hesperetin ve viteksin bitkilerde yaygın olarak bulunan flavonoid'lerdir. Bu çalışmada fisetin, hesperetin ve viteksinin HL60 APL and K562 KML hücreleri üzerindeki sitotoksik, apoptotik ve sitostatik etkilerinin incelenmesi amaçlanmaktadır. Bu üç flavonoid'in APL ve KML üzerindeki etkilerini gösteren herhangi bir çalışma bulunmamaktadır. Bu çalışmada ayrıca tüm genom mikroçip analizi yapılarak fisetin ve hesperetin ile muamele edilen APL ve KML hücrelerinde değişime uğrayan genler ve sinyal iletim yollarının belirlenmesi amaçlanmıştır. Özet olarak, söz konusu flavonoidlerin APL ve KML hücreleri üzerindeki etkilerinin moleküler mekanizmaları ve global gen ifadesindeki değişikliklerin ilk defa gösterilmesi amaçlanmıştır.

Fisetin, hesperetin ve viteksin ile muamele edilen K562 KML ve HL60 APL hücrelerinde doza ve zamana bağımlı olarak hücre canlılığında bir azalma olduğu gösterilmiştir. Her iki hücre tipinde apoptozun tetiklenmesinde en etkili ajanın fisetin olduğu saptanmıştır. Ayrıca, fisetin, hesperetin ve viteksinin her iki hücre tipinde hücre döngüsünün farklı safhaları üzerinde etkili olduğu dolayısıyla sitostatik etkiye sahip olduğu gösterilmiştir.

Sonuç olarak, fisetin ve hesperetinin hücre çoğalmasını inhibe etmeleri, apoptozu tetiklemeleri ve hücre döngüsünün çeşitli safhalarında tutulmalara neden olmalarından dolayı hem KML hem de APL tedavisinde terapötik potansiyellerinin olabileceği düşünülmektedir. Ayrıca, tüm genom mikroçip analizinden elde edilen sonuçlar fisetin ve hesperetin muamelesi sonucu etkilenen biyolojik yolların ve bu yollarla görev alan molekülleri kodlayan genlerin tanımlanmasını sağlamıştır. Bu genlerin hedeflenerek KML ve APL tedavisinde kullanılabileceği düşünülmektedir.

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CHAPTER 1

INTRODUCTION

1.1. Chronic Myeloid Leukemia (CML)

Chronic myeloid leukemia is a myeloproliferative disorder resulting from a balanced reciprocal translocation between the *ABL* (Abelson murine leukemia virus) proto-oncogene on the long arm of chromosome 9 and *BCR* (Breakpoint cluster region) gene on the long arm of chromosome 22, t(9;22)(q34;q11). This translocation results in the generation of a shortened chromosome 22, commonly known as the Philadelphia chromosome (Ph), which encodes the chimeric BCR-ABL1 protein with constitutive kinase activity (Quinta's-Cardama and Cortes 2009). The Ph chromosome is found majority (about 90%) of CML patients (Salesse and Verfaillie 2002). Normally, human *ABL* gene, homologue of the *v-abl* oncogene encoded by the Abelson murine leukemia virus (A-MuLV), codes for a 145 kDa nonreceptor tyrosine kinase that can be localized either in the nucleus or cytoplasm based on the stimuli and is involved in transmission of signals from outside and inside of the cells. Therefore, it affects decisions in terms of apoptosis and cell cycle. Similar to ABL, the 160 kDa BCR protein is ubiquitously expressed and its main biological function remains to be identified though it is known to be involved in several signalling pathways (Deininger, et al. 2000).

Three main BCR-ABL1 transcripts known to be generated based on different breakpoints in BCR gene encode a 210 kDa cytoplasmic fusion protein, p210^{BCR/ABL}, a 230 kDa p230^{BCR/ABL} and a 190 kDa p190^{BCR/ABL}. p210^{BCR/ABL} protein is essential and responsible for phenotypic properties of CML. On the other hand, p230^{BCR/ABL} and p190^{BCR/ABL} are associated with chronic neutrophilic leukemia and acute lymphocytic leukemia, respectively (Melo 1996).

BCR-ABL1 kinase consists of different domains with significant functions (Figure 1.1). The Cap region localized at the N terminus is present in two different isoforms, 1a and 1b, resulting from alternative splicing of first ABL1 exon. BCR-ABL1 also contains a tyrosine kinase domain (SH1) followed by Src-homology-2 (SH2) and SH3 domains. The last exon part includes proline-rich SH3 motifs that function as

binding sites for the SH3 domains of adaptor proteins, a DNA-binding domain, an actin-binding domain, 3 nuclear localization signals, and 1 nuclear export signal, which is responsible for ABL1 subcellular localization in response to environmental factors (Salesse and Verfaillie 2002).

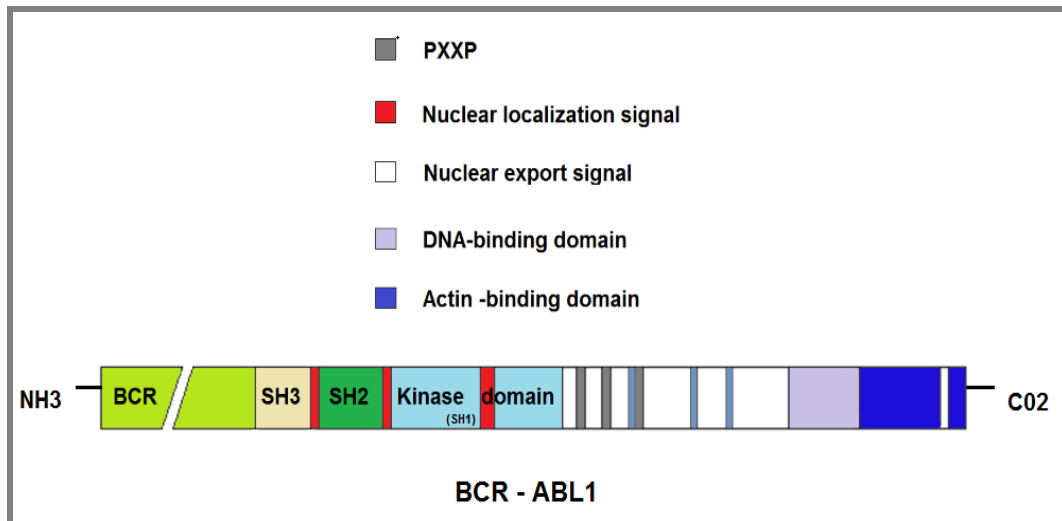


Figure 1.1. Schematic representation of the BCR-ABL1 kinase. The NH2 terminus in BCR-ABL1 kinase is the “Cap” region. PXXP: Proline rich SH3 motifs (Adapted from Quinta’s- Cardama and Cortes 2009)

ABL tyrosine kinase activity is under strict regulation and its SH3 domain is responsible for a critical role in this regulation process. Because, ABL becomes constitutively activated after deletion or positional changes at SH3 domain. The fusion of BCR sequences to the ABL SH3 domain prevents the physiologic suppression of the kinase. Therefore, in contrast to ABL1, BCR-ABL1 has constitutively active tyrosine kinase activity and is localized in the cytoplasm, where it interacts with various proteins involved in the oncogenic pathway (Deininger, et al. 2000, Salesse and Verfaillie 2002).

CML is clinically characterized by distinct stages. In the chronic phase (CP), myeloid progenitor cells display massive expansion without any problem in their differentiation and leave the bone marrow prematurely. This stage is followed by the accelerated phase (AP) and acute leukemic progression phase called blast crisis (BP) with additional cytogenetic abnormalities (Maru 2012). CML has an incidence of 1–2 cases per 100,000 adults, and accounts for 15% of all newly diagnosed adult leukemias (Jabbour and Kantarjian 2014).

1.2. Signaling Pathways Activated by BCR-ABL1

BCR-ABL1 activates various downstream signaling pathways responsible for uncontrolled cell proliferation, growth factor autonomy, reduced apoptosis, altered adhesion to stromal cells and extracellular matrix (Deininger, et al. 2000), and finally CML phenotype develops (Figure 1.2 and Figure 1.3).

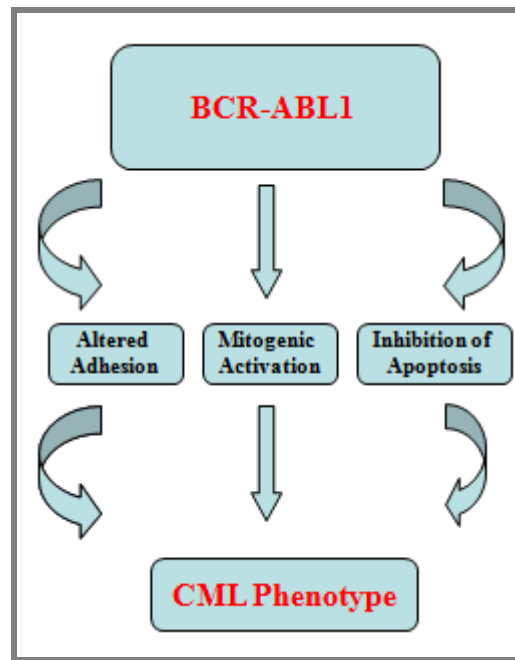


Figure 1.2. Mechanisms involved in the development of CML phenotype (Adapted from Deininger, et al. 2000)

1.2.1. Ras and the MAP kinase pathway

Mutations activating Ras, or alterations in Ras signaling are identified in most human cancers such as leukemias, causing increased cellular proliferation and suppression of apoptosis (Jagani, et al. 2008). Numerous connections between BCR-ABL1 and Ras have been identified. Autophosphorylation of Tyr 177 at BCR part is critical for BCR-ABL1 mediated disease formation since it serves as a binding site for downstream adaptor molecules such as GRB2 (Growth factor receptor-bound protein 2) (Zhang, et al. 2001). GRB2 binds to SOS (Son of sevenless, a guanine nucleotide exchanger of RAS) and stabilizes RAS in its active GTP bound form (Ren 2005). Activated RAS recruits serine/threonine kinase RAF to the cell membrane and RAF

triggers a signalling cascade through the serine/threonine kinases Mek1/Mek2 and Erk, which finally results in the activation of gene transcription such *BCL-2* gene transcription and then prevention of apoptosis (Cahill, et al. 1996). Mutation of Tyr 177 to phenylalanine (Tyr177Phe) was found to prevent GRB2 binding and therefore, BCR-ABL1 mediated RAS activation. Although this mutation does not affect the kinase activity of ABL1, it impairs the malignant transformation of primary bone marrow cultures (Zhang, et al. 2001, Quintana's-Cardama and Cortes 2009).

1.2.2. PI3 Kinase Pathway

PI3 kinase activity is required for the proliferation of BCR-ABL-positive cells. Binding of GRB2 to the phosphorylated Tyr177 residue on BCR-ABL1 via its SH2 domain recruits GAB2 (GRB2-associated binding protein 2), which is in turn phosphorylated by BCR-ABL1 (Sattler, et al. 2002). Phosphorylated GAB2 causes constitutive activation of the phosphatidylinositol 3-kinase (PI3K) by recruiting PI3K via its p85 regulatory subunit (Skorski, et al. 1995). PI3K phosphorylates phosphatidylinositol biphosphate (PIP2) to form phosphatidylinositol triphosphate (PIP3), which acts as a platform for the recruitment of serine/threonine kinase AKT. Phosphorylated AKT regulates the function of many substrates such as MDM-2, caspase-9, mTOR, Bad, and FOXO involved in cell growth, survival, and suppression of apoptosis (Jagani, et al. 2008). Numerous *in vitro* and *in vivo* studies have been performed to show the significance of PI3K/AKT in BCR-ABL1 transformation and leukemogenesis. For instance, treatment of primary CD34⁺ CML cells from acute, chronic and blast crisis phases and BCR-ABL-infected murine bone marrow cells with a PI3K inhibitor, wortmannin, resulted in decreased colony formation (Skorski, et al. 1995). In an *in vivo* study, SCID mice injected with bone marrow cells expressing BCR-ABL1 and dominant negative AKT showed attenuation in leukemia development (Skorski, et al. 1997).

1.2.3. JAK-STAT Pathway

The potential role of the signal transduction and activators of transcription (STAT) factors in leukemia development has been found to be related to their roles in

cell growth, differentiation and apoptosis. Binding of ligand to cytokine or growth factor receptors leads to STAT phosphorylation, dimerization, and subsequent translocation to the nucleus (Yu, et al. 2009). Target genes for STAT include Bcl-xL and Mcl-1 (myeloid leukemia cell differentiation protein) with antiapoptotic functions (Jagani, et al. 2008). Constitutive phosphorylation of STAT1 and STAT5 has been implicated in several CML cell lines and in primary CML cells. STAT5 activation results in suppression of apoptosis through upregulation of Bcl-xL in BCR-ABL1 transformed cells (Horita, et al. 2000). Inhibition of STAT5 via siRNA and antisense oligonucleotide applications resulted in induction of apoptosis in K562 CML cells (Kaymaz, et al. 2013) and it was related to downregulation of STAT5 targets Bcl-xL, cyclinD1, and c-myc at both mRNA and protein levels (Wang, et al. 2011). BCR-ABL1 activates STAT5 both directly and indirectly through activation of JAK2 and the SRC kinases HCK and LYN (Quinta's-Cardama and Cortes 2009).

1.2.4. NF- κ B Pathway

The Nuclear Factor- κ B (NF- κ B) acts as dimers and can be activated by several stimuli such as cytokines, viruses and oxidative stress. Its regulation depends on an inhibitor called the I κ B (inhibitor of κ B), which sequesters NF- κ B to the cytoplasm by binding its nuclear localization signal. NF- κ B only translocates to the nucleus when I κ B is phosphorylated and degraded. Active NF- κ B promotes the transcription of several genes involved in survival/antiapoptosis and angiogenesis (Li and Sethi 2010). In various cancer types, constitutive activation of NF- κ B leads to the development of resistance against apoptosis through upregulation of Bcl-Xl and inhibitors of apoptosis proteins such as IAP1, IAP2, and XIAP (Jagani, et al. 2008). NF- κ B is found to be activated in CML blasts and its activation results in both malignant transformation by BCR-ABL1 and production of growth factors in CML cells (Maru 2012). In a recent study, it has been shown that NF- κ B is activated downstream of BCR-ABL1 in BCR-ABL1 transfected BaF murine cells, LAMA84 human CML cell line and primary CML cells. NF- κ B inhibition by I κ B kinase inhibitor AS602868 resulted in induction of apoptosis in T315I (conversion of tyrosine at 315 residue into isoleucine, resistant against all currently used BCR-ABL1 inhibitors) expressing cells (Lounnas, et al. 2009).

1.2.5. Integrin Signalling

Adhesion to stromal cells and extracellular matrix is a way to regulate cell proliferation in a negative manner. However, CML cells have defective adhesion properties to escape from this regulation. The interaction between stroma and progenitor cells is mediated by β -integrins. CML cells possess a variant form of β -integrin that is not found in normal progenitor cells and inhibit adhesion. Normally, after binding to their receptors, integrins initiate a signaling cascade responsible for the inhibition of cell proliferation. Therefore, the transmission of signals that normally impair cell proliferation is defective in CML cells (Deininger, et al. 2000). Crk1 is one of the most common tyrosine phosphorylated protein in BCR-ABL1 positive cells and involved in the regulation of cell migration and integrin-mediated cell adhesion in association with other focal adhesion kinases such as paxillin and focal adhesion kinase FAK (Uemura and Griffin 1999, Deininger, et al. 2000).

1.2.6. MYC Pathway

The protooncogene MYC, another nuclear target of BCR-ABL1, is activated by the SH2 domain of BCR-ABL1 (Cilloni and Saglio 2012). However, molecular pathway between the SH2 domain and MYC remains to be elucidated. MYC is generally overexpressed in blast crisis compared to chronic phase, which is linked to disease progression (Cilloni and Saglio 2012, Sawyers, et al. 1992). BCR-ABL transformation or leukemogenesis can be inhibited by inhibiting MYC via antisense oligonucleotides or dominant-negative constructs (Sawyers, et al. 1992).

1.2.7. BCR-ABL Inhibition of the PP2A Phosphatase

The tumor suppressor serine/threonine protein phosphatase, PP2A, is found to be inactivated by the PP2A inhibitor, SET, which is upregulated by BCR-ABL1 mediated way at transcription level (Neviani, et al. 2005). PP2A inactivation causes hyperphosphorylation and inactivation of proapoptotic Bad whereas activating some substrates such as AKT and ERK involved in survival and proliferation signaling (Jagani, et al. 2008). Reactivation of PP2A in several imatinib-sensitive and imatinib-

resistant CML cell lines results in decreases in BCR-ABL1 levels through mediating its degradation (Salas, et al. 2011). Thus, functional inactivation of PP2A is essential for BCR-ABL-mediated leukemogenesis.

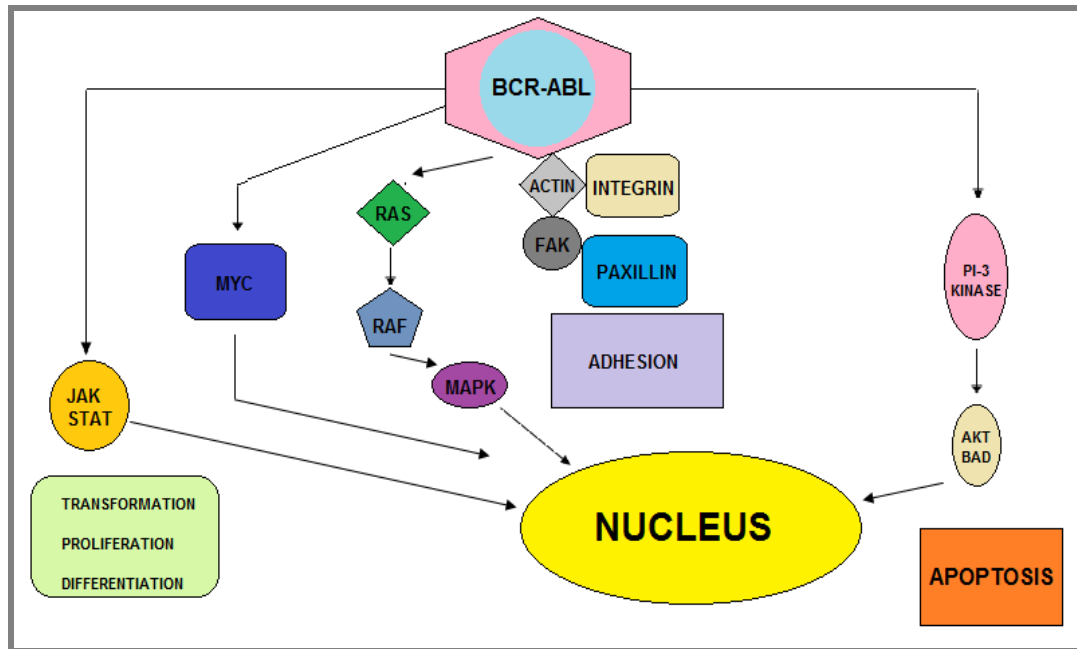


Figure 1.3. Downstream signalling pathways activated by BCR-ABL (Adapted from Salesse and Verfaillie 2002). Activation of these pathways are responsible for increased proliferation and differentiation, decreased apoptosis and adhesion

1.3. Treatment Options for CML

X-radiation to spleen and the usage of conventional drugs such as hydroxyurea were the first strategies to treat CML patients, which restricted the expansion of myeloid tissue and increased the quality of life during CP of CML (Baccarani, et al. 2006). However, cytogenetic responses are rare. Moreover, AP and BP onsets are not delayed. The first important breakthrough in CML therapy was the application of allogeneic stem cell transplantation (allo SCT), because 50% of patients under cure showed Philadelphia-negative and BCR-ABL negative pattern. However, finding appropriate donor and the development of graft versus host disease were the drawbacks of allo SCT (Pavlu, et al. 2011). The introduction of human recombinant interferon- α into CML therapy was the second breakthrough, providing a complete cytogenetic remission in 15% to 30% of patients and a significant survival advantage. On the other

hand, it is not well tolerated by some patients and its combination with other drugs increases toxicity (Talpaz, et al. 2013). Widespread presence of BCR/ABL oncoprotein in patients with CML and developing structural studies have facilitated the design of therapeutics targeting the tyrosine kinase activity of BCR-ABL and all mentioned treatments were displayed after the development of tyrosine kinase inhibitors (TKIs) (Jabbour and Kantarjian 2014).

1.3.1. Imatinib Mesylate (STI571)

Imatinib mesylate (Gleevec, Novartis Pharmaceutical Corporation, NJ), was the first TKI used in a clinical application (Jabbour and Kantarjian 2014) after its efficacy was proven by multiple *in vitro* (including CML cell lines and primary CML cells), *in vivo* (including mice with 32D^{BCR/ABL}-generated tumors) and animal toxicity profile studies (Salesse and Verfaillie 2002). Normally, ATP binds to ATP-binding pocket in the BCR-ABL SH1 domain and then, BCR-ABL phosphorylates tyrosine residues on its substrates involved in several signal transduction pathways. Imatinib mesylate binds to BCR-ABL kinase domain (Figure 1.4), which is an inactive conformation in a pocket, thus tyrosine kinase activity fails due to the inhibition of phosphorylation of BCR-ABL substrates (Fausel 2007). In conclusion, proliferative signals are prevented and CML cells apoptosis is induced. It also inhibits the platelet-derived growth factor receptor (PDGFR) and c-kit tyrosine kinase as well as BCR-ABL (Druker and Lydon 2000).

Imatinib was approved by The Food and Drug Administration (FDA) as first-line treatment for newly diagnosed CML in 2002 based on the results of an International Randomized Study (IRIS) initiated in 2000. This study compared 400 mg single dose imatinib with IFN alpha plus cytarabine in newly diagnosed CML patients in CP (Sacha 2013). However, only 55% of patients registered in this study remained on therapy during the 8-year follow up time due to the development of intolerance or resistance to imatinib and the presence of the minimal residual disease (MRD) (Deininger, et al. 2009). These outcomes lead to the design of second generation TIKs such as nilotinib and dasatinib and third generation TIKs such as bosutinib and ponatinib.

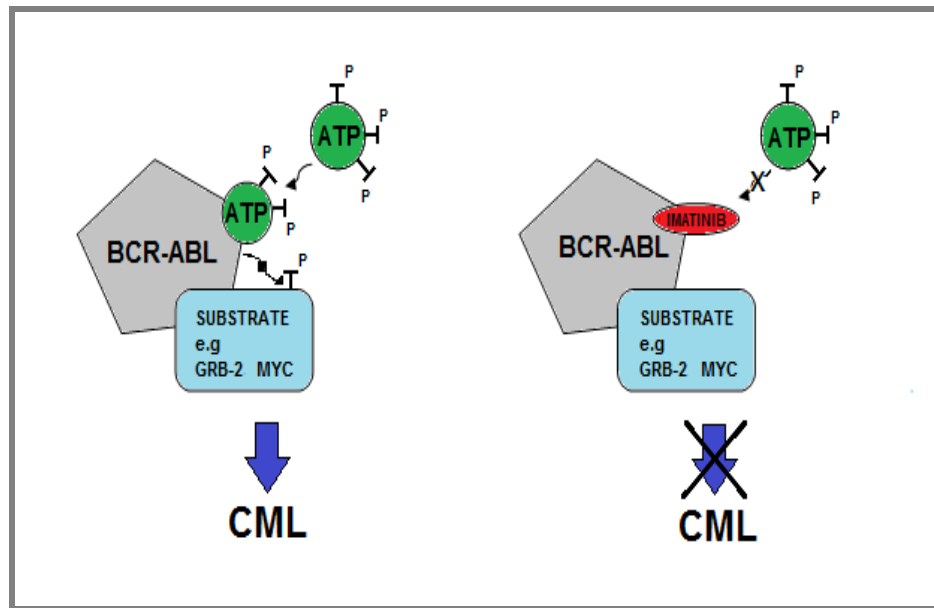


Figure 1.4. Mechanism of action of tyrosine kinase inhibitors

1.3.2. Nilotinib (AMN107)

Nilotinib (Tasigna, Novartis Pharmaceutical Corporation, NJ), a structural analog of imatinib, was designed to improve imatinib's solubility and oral bioavailability by incorporating a *N*-methylpiperazine moiety (Melo and Chuah 2008) and approved by FDA in 2010. It binds to ATP binding pocket on BCR-ABL 50 times more potent than imatinib *in vitro* (Jabbour and Kantarjian 2014). Similar to imatinib, it also inhibits PDGFR and c-kit, but not Src family kinases (Melo and Chuah 2008). Nilotinib inhibits proliferation of wild type BCR-ABL cell lines and most of the BCR-ABL mutants, except the T315I mutation, 10 to 50 times more potent than imatinib (Weisberg, et al. 2005). It is also superior to imatinib in prolonging survival of mice injected with wild type and mutant BCR-ABL (Weisberg, et al. 2005).

The effects of nilotinib on newly diagnosed CML patients have been investigated as compared to imatinib in a large, international, randomized study (ENESTnd) in which two doses of nilotinib (300 or 400 mg twice daily) were compared with imatinib 400 mg once daily. The results of this study indicated that both doses of nilotinib achieved faster major molecular response (MMR) and less progression to AP or BP (Larson, et al. 2012).

1.3.3. Dasatinib (BMS-354825)

Dasatinib (BMS-354825, Sprycel; Bristol Myers Squibb) is a multi-target kinase inhibitor approved in 2010 by FDA and 350 times more potent than imatinib *in vitro*. It has the ability to bind to imatinib resistant active conformation of the kinase domain and to inhibit the proliferation of wild type and most BCR-ABL mutant cell lines except T315I mutation (Melo and Chuah 2008). Dasatinib was also found to inhibit leukemic cell growth and prolong survival of mice having wild type and mutant BCR-ABL (Shah, et al. 2004).

A randomized phase III study (DASISION) compared dasatinib 100 mg once daily to imatinib 400 mg once daily in newly diagnosed CML patients, indicating that dasatinib gives more rapid and effective responses at early time points compared to imatinib (Jabbour, et al. 2014).

1.3.4. Bosutinib (SKI-606)

Bosutinib (Bosulif®, Pfizer, New York, NY) is a third generation TKI approved by FDA in 2012 in the United States for the treatment of imatinib sensitive and resistant CP, AP and BP CML regardless of the presence or absence of ABL kinase domain mutations, with the exception of T315I (Hill, et al. 2014). It is a dual inhibitor of BCR-ABL and Src kinases and binds to both active and intermediate conformations of BCR-ABL (Shen, et al. 2014). Unlike dasatinib, it does not inhibit PDGFR and c-kit and is well tolerated (Hill, et al. 2014).

The results of Bosutinib Efficacy and Safety in Newly Diagnosed CML trial (BELA) has displayed that it has clinical activity, manageable toxicity and safety in newly diagnosed CML patients compared to imatinib (Gambacorti-Passerini, et al. 2014).

1.3.5. Ponatinib (AP24534)

Ponatinib (Iclusig tablets, ARIAD Pharmaceuticals, Inc.) was approved by FDA in 2012 for the treatment of adult patients with CP, AP, or BP CML that are resistant or intolerant to previous TKI therapy based on the results of a phase II PACE study

(Cortes, et al. 2013, Shamroe, et al. 2013). Ponatinib is a pan-BCR-ABL TKI with potent activity against BCR-ABL, as well as other kinases such as PDGF, c-kit and Src kinases. It is known to be 520 times more potent than imatinib and to compete with ATP and bind to inactive conformation of BCR-ABL like imatinib and nilotinib. Unlike other BCR-ABL TKIs, ponatinib has the ability to overcome resistance caused by the T315I mutation (Jabbour and Kantarjian 2014, Shamroe, et al. 2013).

1.3.6. Novel TKIs

DCC-2036 is a novel TIK with a different working principle that blocks the switch pocket involved in conformational regulation of the kinase domain rather than the ATP pocket. Up to date, it has been shown to inhibit all mutant forms of BCR-ABL *in vitro* (Eide, et al. 2011). Thiotanib, a novel TKI, selectively targets BCR-ABL and induces growth inhibition, cell cycle arrest and apoptosis in CML cells (Fan, et al. 2014). Radotinib has been shown to possess significant effects in preclinical and phase I/phase II clinical trials that include CML patients with resistance and/or intolerance to BCR-ABL1 tyrosine kinase inhibitors (Kim, et al. 2014). HS-438, inhibited the expression of BCR-ABL signaling pathways in wild-type BCR-ABL (BaF3/WT) cells as well as T315I-mutated BCR-ABL (BaF3/T315I) cells with resistance to imatinib (Yun, et al. 2014).

1.3.7. Hematopoietic Stem Cell Transplantation

Allo SCT was historically the first option for patients with newly diagnosed younger CP CML. However, this situation has changed significantly since TIKs were introduced. Allo SCT has become more important for patients who progress into AP/BP. On the other hand, Allo SCT still remains an important therapeutic option for CML CP in the following situations: patients who could not respond to at least two TKIs and potentially patients having the T315I mutation after a trial of ponatinib therapy. Patients with AP/BP CML may be treated with TIKs initially, especially dasatinib or ponatinib, to reduce the CML area and be considered for early allo SCT. However, at present, allo SCT is the only curative therapy for AP and BP patients (Velmand, et al. 2013, Jabbour and Kanterjian 2014).

1.3.8. Alternative Treatment Approaches for CML

Targeting molecules involved in downstream signaling pathways of BCR-ABL such as Ras and the MAP kinase pathways might provide a synergistic antiproliferative and pro-apoptotic effect when combined with TIKs in CML. Prenylation, important posttranslational modification for Ras function, is catalyzed by farnesyltransferase (FT). Therefore, FT inhibitors such as tipifarnib have displayed antileukemic activity in CML. In a phase I study including imatinib-resistant CML patients, the combination of imatinib and tipifarnib was found to be active and well tolerated (Cortes, et al. 2007). PD184352, MEK1/2 inhibitor, killed drug resistant CML cells and blocked tumor growth in mice when combined with nilotinib (Packer, et al. 2011).

Targeting Histone deacetylases (HDAC) is another way to cope with CML. SAHA, histone deacetylase inhibitor, was found to act synergistically with imatinib on K562 CML cells and induce apoptosis by activating caspase-3, -8 and PARP and downregulating JAK2 and STAT5 (Liu, et al. 2012).

Bortezomib (PS-341, Velcade; Millennium Pharmaceuticals), a proteasome inhibitor, prolonged life span and reduced tumor growth in murine model of CML when combined with low dose of imatinib. Moreover, it increased the apoptotic effects of imatinib through inhibition of Bcl-2 and activation of caspases (Hu, et al. 2009). Carfilzomib, next generation proteasome inhibitor, decreased proliferation and induced apoptosis in imatinib-sensitive and -resistant CML models by decreasing ERK signalling (Crawford, et al. 2014).

Flavopiridol (L86-8275; National Cancer Institute), cyclin dependent kinase inhibitor, together with imatinib resulted in mitochondrial damage, caspase activation and apoptosis in CML but not in BCR-ABL negative leukemic cell lines (Yu, et al. 2002).

DNA methyltransferase and heat shock protein 90 inhibitors and PP2A activators are the other molecules that can be used in order to manage CML (Melo and Chuah 2008).

1.4. Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) is a heterogeneous and complex disease observed in the precursors of hematopoietic cell lineages that undergo clonal transformation through the acquisition of chromosomal alterations (inversions, translocations etc.) together with multiple gene mutations (Rubnitz, et al. 2010). Such alterations produce proteins, also called fusion proteins, with aberrant functions. One of these proteins is generally a transcription factor whereas other protein has variable function involved in the regulation of cell survival and apoptosis (Alcalay, et al. 2001). Finally, various critical signalling pathways become dysregulated and hematopoietic precursors gain proliferative and survival advantage while their differentiation and apoptosis are impaired (Dash and Gilliland 2001). These neoplastic leukemic cells accumulate in the bone marrow and take the place of normal bone marrow cells, causing decreases in the number of platelets, red blood cells and white blood cells (Shipley and Butera 2009). In conclusion, there is no way to define a single disease causing event in AML although several cytogenetic, molecular and epigenetic alterations have been identified. Unlike CML, there is no single step process for AML pathogenesis. Based on different chromosomal translocations observed in AML cells, there are several subtypes of AML according to WHO organization (Table 1.1) (Vardiman, et al. 2009). The classification of AML is also based on additional biological features such as morphology, cytogenetics, immunophenotype and clinical data.

Table 1.1. Classification of AML based on WHO organization (Vardiman, et al. 2009)

Acute myeloid leukemia with recurrent genetic abnormalities • AML with t(8;21)(q22;q22); RUNX1-RUNX1T1 • AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 • APL with t(15;17)(q22;q12); PML-RARA • AML with t(9;11)(p22;q23); MLLT3-MLL • AML with t(6;9)(p23;q34); DEK-NUP214 • AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1 • AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1 • Provisional entity: AML with mutated NPM1 • Provisional entity: AML with mutated CEBPA Acute myeloid leukemia with myelodysplasia-related changes Therapy-related myeloid neoplasms Acute myeloid leukemia, not otherwise specified • AML with minimal differentiation • AML without maturation • AML with maturation • Acute myelomonocytic leukemia • Acute monoblastic/monocytic leukemia • Acute erythroid leukemia ○ Pure erythroid leukemia ○ Erythroleukemia, erythroid/myeloid • Acute megakaryoblastic leukemia • Acute basophilic leukemia • Acute panmyelosis with myelofibrosis Myeloid sarcoma Myeloid proliferations related to Down syndrome ○ Transient abnormal myelopoiesis ○ Myeloid leukemia associated with Down syndrome Blastic plasmacytoid dendritic cell neoplasm
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1.5. Acute Promyelocytic Leukemia (APL)

Acute promyelocytic leukaemia (APL), a distinct variant of AML, is different from other types of AML with its molecular biology and clinical outcomes. APL is characterized by abnormal accumulation of immature granulocytes (promyelocytes) in the bone marrow and blood stream and accounts for 10% of cases of AML (Lo-Coco, et al. 2014). It evolves very rapidly and causes sudden hemorrhages, thus it is extremely malignant. APL is characterized by specific chromosomal translocations between the retinoic acid (RA) receptor α (RAR α) gene on chromosome 17 and any other gene to create a variety of X-RAR α fusions. In the majority of APL cases, translocation, t(15;17), between the retinoic acid receptor-alpha (RAR α) gene on chromosome 17 and the promyelocytic leukaemia (PML) gene on chromosome 15 is very common. The resultant PML-RAR α fusion protein plays critical role in the pathogenesis of APL (de Thé, et al. 2012).

There are two proposed mechanisms to explain how t (15;17) is formed and initiates APL, which are *de novo* APL and therapy-related APL. The reasons for *de novo* APL formation are largely unknown, however, endogenous DNA damage and errors in DNA recombination are thought to be possible mechanisms (Lo-Coco, et al. 2014). Most information about t(15;17) formation can be obtained from therapy-related APL. Some anti-cancer drugs used for a primary disease have been shown to induce APL. For instance, agents such as daunorubicin and mitoxantrone, targeting topoisomerase II alpha, were displayed to have leukemogenic effects by inducing direct DNA damage or sister chromatid exchanges (SCE) or chromosomal translocations (Mays, et al. 2010, Hasan, et al. 2008).

When retinoids (vitamin A and its derivatives) bind to their nuclear receptors, RAR α (retinoic acid receptor α) and RXR (the retinoid X receptors), they regulate embryogenesis, cell differentiation and apoptosis (Lo-Coco, et al. 2014). In the absence of ligand, RAR α and RXR become dimerized and cause transcriptional repression by forming complexes with histone deacetylases (HDACs). Upon binding of retinoic acid (RA) to RAR α -RXR complex, conformational changes occur in the complex and HDACs are released and histone acetyl transferases (HATs) are recruited. As a result of acetylation of histones, target genes involved in cell differentiation are activated. The PML-RAR α fusion protein, an altered retinoic acid receptor, is a potent recruiter of transcriptional repressors such as DNA methyltransferases (DNMTs) and HDACs that

are resistant to derepression by physiologic concentrations of RA. Therefore, this fusion protein represses the RARE (retinoic acid response element)-containing genes mainly involved in differentiation. The leukemic cells with t(15;17) are arrested in the immature, promyelocyte stage, which causes uncontrolled proliferation of the promyelocytic blasts (Lo-Coco, et al. 2014) (Figure 1.5).

Although PML-RAR α is the driving event to initiate typical APL, additional genetic/epigenetic changes are required to accelerate APL phenotype. FMS-related tyrosine kinase 3 (FLT3) gene or trisomy 8 has been shown to be responsible for APL progression in transgenic mice model (Kogan 2007). In a study with mouse and human APL genome, a point mutation in *JAK1* gene or a deletion in a histone demethylase has been identified (Wartman, et al. 2011). Mutations in several oncogenes such as *K-RAS* has been found to be together with PML-RAR α (Chan, et al. 2006).

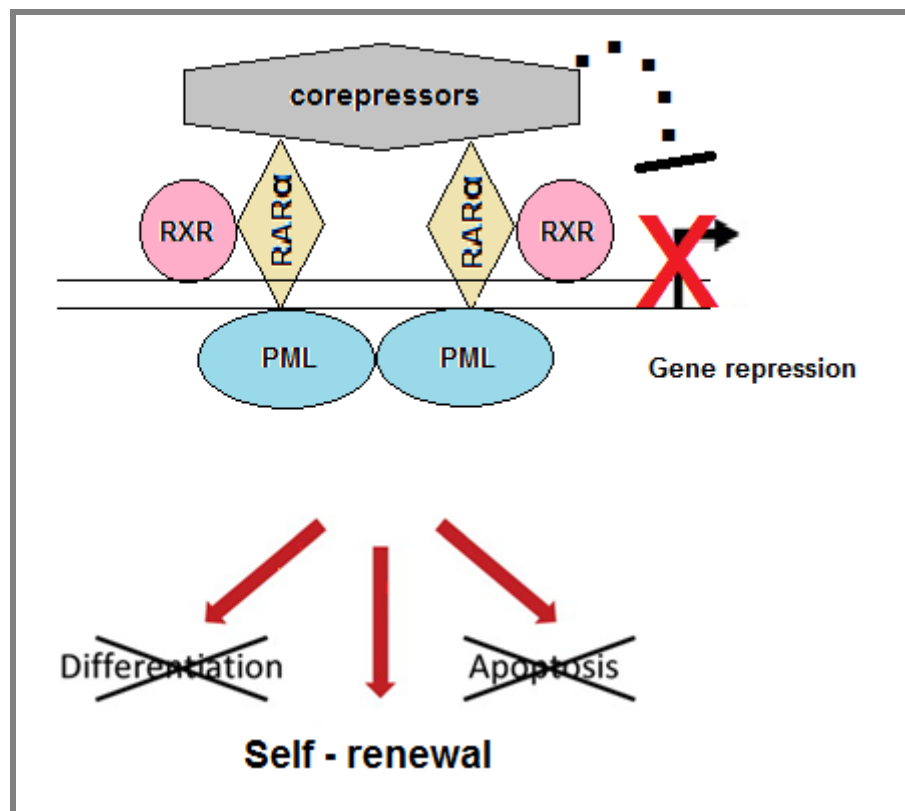


Figure 1.5. Schematic representation of PML-RAR α function for the development of APL

1.5.1. Targets of PML-RAR α

The balance between three transcription factors, PU.1, C/EBP α and GATA-1 are very important to determine the differentiation of hematopoietic stem cells (HSCs) into various myeloid lineages. In a recent study, PU.1 was found to direct PML-RAR α binding to RARE sites by forming PML-RAR α /PU.1 complex through protein-protein interactions (Wang, et al. 2010). Therefore, PU.1 (a main regulator of myeloid differentiation) targeted genes mainly involved in myeloid differentiation were blocked at the transcription level. Moreover, PML-RAR α inhibits PU.1 at the transcriptional level, resulting in repression of PU.1 targeted genes such as CD45, the granulocyte/macrophage colony stimulating factor receptor (GM-CSFR), myeloperoxidase, lysozyme, and neutrophil elastase involved in myeloid differentiation and function (Lo-Coco, et al. 2014).

1.6. Treatment Concepts of APL

Historically, newly diagnosed APL patients were treated with daunorubicin and cytosine arabinoside (ara-C) that have curative potential and induce long lasting remission. However, the development of coagulopathy due to the release of azure granules in the malignant cells resulted in patient deaths (Lengfelder, et al. 2005). Therefore, there was a need for a more effective therapy because of less efficacy of chemotherapy alone on APL.

1.6.1. All-Trans Retinoic Acid (ATRA)

Understanding of the critical role of PML-RAR α in APL development makes it a potential target for APL treatment. In 1980s, the introduction of All-Trans Retinoic Acid (ATRA) into APL therapy improved the outcomes of *de novo* APL patients. ATRA targets RAR α part of PML-RAR α and has a role in directing PML-RAR α degradation in a proteasome-dependent way. Therefore, ATRA induces irreversible differentiation of leukemic promyelocytes, resulting in their apoptosis (Gianni, et al. 2000). Although complete hematological remission rate is very high with ATRA-monotherapy in newly diagnosed APL patients, ATRA alone can not induce stable

remission due to a relatively high recurrence rate (Mi, et al. 2012). The combination therapy with chemotherapy was found to have superior effects with lower recurrence and better survival rates (Adès, et al. 2010). Thus, the combination of ATRA and chemotherapy is currently considered as the standard protocol for newly diagnosed APL.

1.6.2. Arsenic Trioxide (ATO)

Arsenic Trioxide (ATO) functions by binding to PML moiety of PML-RAR α oncoprotein, causing its proteasome-dependent degradation and resulting in partial differentiation and induction of apoptosis of leukemic promyelocytes (Mi, et al. 2012, Mi 2011). Several studies confirmed that ATO alone was a very effective regimen inducing complete hematological and, in some cases, molecular responses in APL patients. Furthermore, it increased overall survival in both relapsed and newly diagnosed APL patients (Ghavamzadeh, et al. 2006). As a comparison, it could be said that ATRA was a first line therapy for newly diagnosed patients, whereas ATO was standart treatment for relapsed patients who were previously treated ATRA or chemotherapy. The working principles of ATRO and ATO for APL are explained in Figure 1.6.

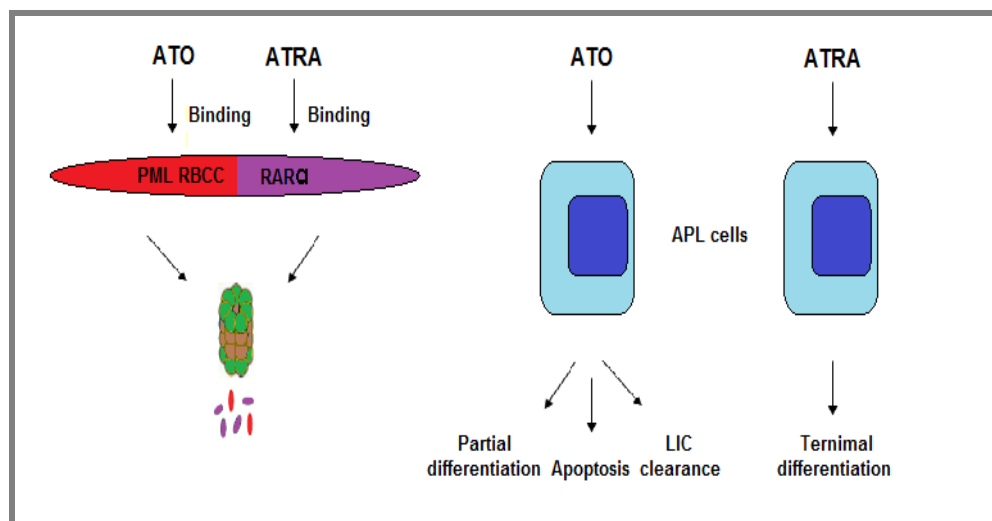


Figure 1.6. The working principles of ATRO and ATO for APL (Adapted from Mi, et al. 2012). LIC: Leukemia initiating cells

1.6.3. ATRA/ATO Combination Therapy

There are several logical reasons to consider ATRA/ATO combination for the therapy of newly diagnosed APL patients: 1. Both of them target PML-RAR α and regulate distinct but related signalling pathways involved in differentiation/apoptosis, 2. Cross-resistance does not occur between them, 3. Their combination increased survival and caused even complete removal of APL in a mouse model (Jing, et al. 2001, Lallemand-Breitenbach, et al. 1999, Zheng, et al. 2005). A randomized clinical study on newly diagnosed APL patients revealed that ATRA/ATO combination gave much better results in terms of complete remission and disease free survival compare to either agent alone (Mi 2011).

1.6.4. Monoclonal Antibody Treatment

As an anti-CD33 monoclonal antibody, gemtuzumab ozogamicin (GO), was approved by the FDA in 2000 for the treatment of older patients with relapsed APL. It is an antibody-drug conjugate made up of anti-human CD33 antibody and an antitumor antibiotic, calicheamicin. Calicheamicin induces cell death by binding to DNA and forming double strand breaks. CD33 is commonly expressed on the surface of APL cells. The internalization of CD33 after antibody binding makes this complex entrance into leukemia cells effective (Takeshita 2013). In a study, the clinical efficacy of GO for patients of relapsed APL has been reported (Lo-Coco, et al. 2004) in which most of the registered patients with molecular relapse achieved molecular remission.

1.6.5. Hematopoietic Stem Cell Transplantation

Stem cell transplantations (either allogeneic or autologous) are not currently recommended for patients who have had their first complete remission. Because, most of the patients with untreated APL have an excellent outcome with ATRA and chemotherapy. Autologous transplantation is effectively used in patients with relapsed APL after ATRA treatment and achieved second molecular remission. However, allogeneic transplantation has risks because of the higher transplantation-related mortality (de Botton, et al. 2005). In a study, autologous transplantation was deployed to

be associated with superior clinical outcomes in patients with relapsed APL as compared to ATO and ATRA/ATO therapies (Thirugnanam, et al. 2009).

1.7. Dietary Antioxidants

Recently, natural dietary substances found in fruits, vegetables, and herbs have gained considerable attention as chemopreventive and chemotherapeutic agents (Khan, et al. 2008) due to their non-toxic nature and usage for a long time without any adverse effect. One of the compounds found in plants is flavonoids, naturally occurring polyphenolic compounds, exerting several biological activities such as antioxidant, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, and antiviral effects (Sak 2014). Their contribution to cancer prevention and treatment is the most investigated concept because of two reasons: 1. They are safe and easily obtained, 2. They are toxic to cancer cells (depending on the flavonoid of interest) without any significant negative effects on normal cells (Sak 2014). They have been reported to prevent the initiation, promotion and progression of cancer by making changes in various signaling pathways involved in cell proliferation, differentiation, apoptosis, angiogenesis and metastasis. Thus, flavonoids (both natural and synthetic analogs) are being investigated for their potentials in cancer therapy (Ravishankar, et al. 2013).

Flavonoids can be found either as free aglycones or glycosidic conjugates. Chemically, they are polyphenolic with a phenyl benzopyrone structure (C6–C3–C6) classified mainly into flavones, flavonols, isoflavones, flavonols, flavonones, flavononols and chalcones (Middleton, et al. 2000) (Figure 1.7).

The differences in biological characteristics of leukemias and development of resistance in most cases are responsible for unique therapeutic challenges. Although several treatment options exist for CML and APL as described, they are not cured effectively and the development of drug resistance is common. Thus, it is important to develop novel treatment approaches in order to improve outcome. At present, several plant-derived compounds have produced for clinical uses to treat hematological cancers. These include vinblastine, vincristine, topotecan, irinotecan, etoposide and paclitaxel (Lucas, et al. 2010). Moreover, there are several plant derived agents that are under clinical trials and preclinical investigations.

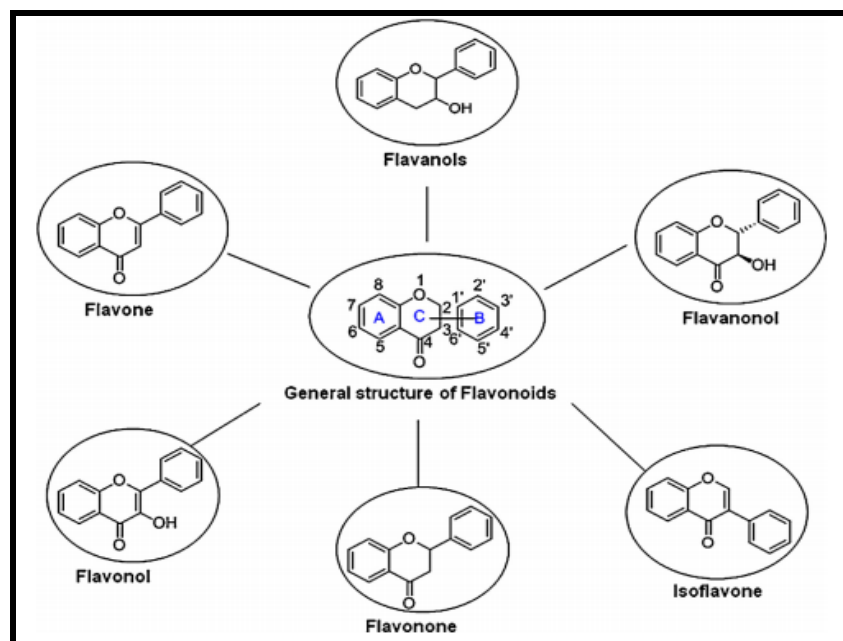


Figure 1.7. Different groups of flavonoids and their chemical structure (Ravishankar, et al. 2013)

1.8. Fisetin (3, 3', 4', 7-tetrahydroxyflavone)

Fisetin (3,3',4',7-tetrahydroxyflavone) (Figure 1.8), a naturally occurring bioactive flavonol molecule, found in fruits and vegetables such as strawberry, apple, persimmon, grape, onion, and cucumber has been reported to be a promising novel antioxidant with its potential as a chemopreventive/chemotherapeutic agent (Khan, et al. 2013).

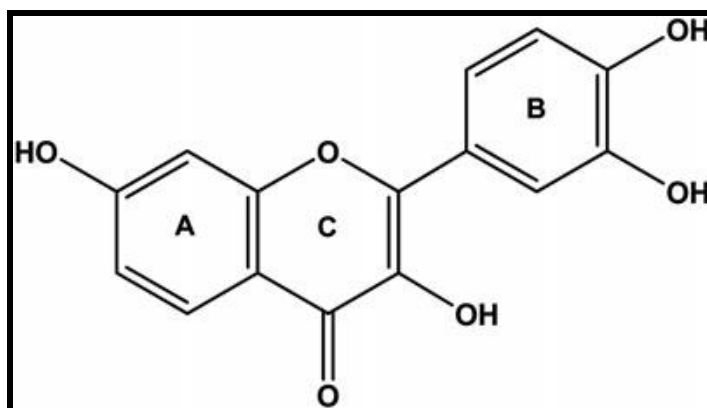


Figure 1.8. The structure of fisetin (Khan, et al. 2013)

Fisetin has been shown to possess various biological activities as well. In earlier studies, fisetin was identified as an antimicrobial agent and found to prevent oxidative-stress related neuronal cell death (Ishige, et al. 2001). Fisetin also stimulated nerve cell differentiation and enhanced memory via Erk activation (Maher, et al. 2006). Moreover, fisetin was shown to have anti-inflammatory activity in brain microglia and could be used as anti-inflammatory agent (Zheng, et al. 2008). Fisetin is also considered as microtubule stabilizer and antimitotic (Touil, et al. 2009).

1.8.1. Fisetin and Cancer: Literature Overview

In vitro and *in vivo* studies have investigated the effects of fisetin on several cancer types.

Fisetin triggered apoptosis in HCT-116 colon cancer cells at concentrations of 5-20 μ M by activating both-death receptor and mitochondrial pathways, resulting in activation of the caspases. Fisetin treatment resulted in decreases in anti-apoptotic Bcl-Xl and Bcl-2 while increasing apoptotic Bak and Bid proteins. Also, fisetin increased p53 levels that leads to Bax translocation to the mitochondria and then apoptosis (Lim do and Park 2009). Fisetin caused the inhibition of cell cycle progression in HT-29 colon cancer cells by decreasing the activities of cell cycle regulator proteins including cyclin-dependent kinases such as cdk-2 and -4 and several cyclins (Lu, et al. 2005). In a recent study, fisetin inhibited Wnt/ β -catenin signaling which was associated with decreased expression of β -catenin target genes such as cyclin-D1 and MMP-7 (matrix metalloproteinase-7) (Suh, et al. 2009).

Fisetin sensitized prostate cancer cells (DU145, PC-3 and LNCaP cells) to TRAIL (tumour necrosis factor-related apoptosis-inducing ligand)-mediated apoptosis. Combination of TRAIL and fisetin increased caspase-8 and caspase-3 activation and disrupted mitochondrial membrane potential (Szliszka, et al. 2011). Fisetin also induced apoptosis in LNCaP cells via activation of caspase-cascade and cell cycle arrest at G0/G1 without any anti-proliferative effect on normal prostate epithelial cells (Khan, et al. 2008). Fisetin could also inhibit the adhesion, migration and invasive behavior of cancer cells. Fisetin had antimetastatic property through the inhibition of PI3K/Akt and JNK signaling together with downregulation of MMP-2/9 in PC-3 cells (Chien, et al. 2010).

Fisetin exerted its apoptotic, anti-proliferative and anti-invasive effects in chemoresistant AsPC-1 pancreatic cancer cells by modulating DR3 receptor mediated down regulation of NF- κ B signaling pathway (Murtaza, et al. 2009). Fisetin treatment resulted in the downregulation of genes such as antiapoptotic BCL2L1 and genes involved in cell invasion, proliferation, and metastasis such as NF- κ B and MMP-9. The upregulated genes including p21, p16 and I κ B α , were related to cell cycle control, invasion and metastasis (Murtaza, et al. 2009). Based on these data, it could be said that NF- κ B/MMP signalling is an important target of fisetin.

Fisetin inhibited A549 lung cancer cell invasion, adhesion and migration by decreasing phosphorylation of ERK1/2 and downregulating the expression of MMP-2 at both the protein and mRNA levels. Fisetin inhibited activation and nuclear translocation of NF- κ B, upstream regulator of MMP-2 (Liao, et al. 2009). Fisetin decreased the viability and clonogenicity of A549 lung cancer cells through the modulation of AMP-activated protein kinase (AMPK)/Akt/mammalian target of rapamycin (mTOR) signaling (Khan, et al. 2012). In an *in vivo* study, fisetin protected B(a)P-exposed mice against B(a)P-induced lung carcinogenesis by reducing histological lesions (Ravichandran, et al. 2011). Lewis lung carcinoma-established mice were treated with fisetin, resulting in decreases in tumor growth due to its anti-angiogenic activity (Touil, et al. 2011). This effect increased when fisetin was combined with cyclophosphamide.

Cervical cancer cells were found to undergo apoptosis after fisetin treatment, which was based on ERK1/2- mediated activation of caspase-8/caspase-3 pathway (Ying, et al. 2012). Fisetin prevented cervical cancer cells from migrating by repressing urokinase plasminogen activator (uPA) via suppression of NF- κ B pathway (Chou, et al. 2013).

The antiproliferative and apoptotic effect of fisetin was also studied on T24 and E7 bladder cancer cells. p53 and p21 proteins were found to increase while the levels of cyclin D1, cyclin A, CDK4 and CDK2 were increased after fisetin treatment, which induces cell cycle arrest. In addition, fisetin induced apoptosis by increasing the expression of Bax and Bak but decreasing the levels of Bcl-2 and Bcl-xL. Finally, it triggered mitochondria mediated apoptotic pathway (Li, et al. 2011). Same mechanisms were responsible for prevention of bladder cancer development in a mouse model (Li, et al. 2014).

In U266 multiple myeloma cells, fisetin displayed cytotoxicity and induced apoptosis via caspase-3 activation, downregulation of Bcl-2 and upregulation of Bax,

Bim and Bad. Fisetin also stimulated reactive oxygen species (ROS) formation and decreased Akt phosphorylation (Jank, et al. 2012). Fisetin had cytotoxic effect on human HL60 APL cells and induced apoptosis via activation of caspase-3, induction of Bax and suppression of Mcl-1 (Lee, et al. 2002). Fisetin triggered apoptosis in U967 leukemic cells via both caspase and calpain-dependent ways (Monasterio, et al. 2004).

1.9. Hesperidin and Hesperetin

Citrus species have attracted significant attention for their flavonoid content which is thought to be promising in cancer therapy as both chemopreventive and chemotherapeutic agent. Hesperidin and hesperetin are the most important examples of these flavonones found in citrus fruits such as lemon and orange. They have a wide range of pharmacological properties including antioxidant, anti-inflammatory and anticancer effects (Benavente-Garcia and Castillo 2008).

Hesperidin is a flavonone glycoside and hesperetin (3',5,7-trihydroxy-4-methoxyflavonone) is aglycone form of hesperidin (Figure 1.9). Hesperidin taken with diet is converted to hesperetin via deglycosylation by intestinal bacteria before absorption. Therefore, hesperidin could be a pro-drug, which is metabolized to hesperetin.

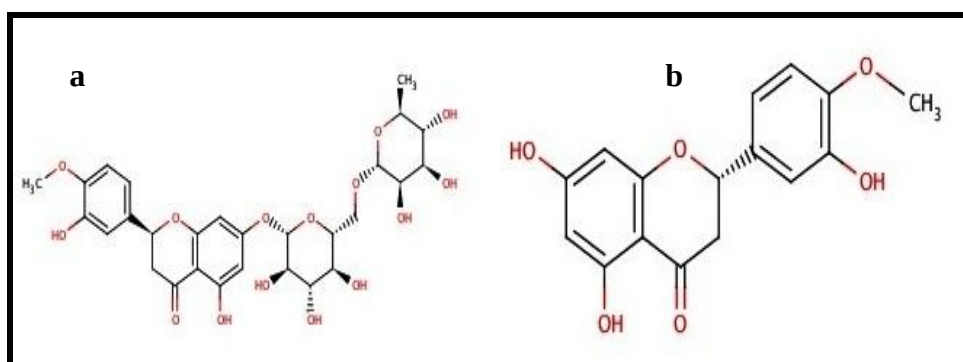


Figure 1.9. The chemical structures of hesperidin (a) and hesperetin (b) (Source: Santa Cruz Biotechnology, Inc.)

1.9.1. Hesperidin and Hesperetin in Cancer: Literature Overview

Apoptotic effect of hesperidin was investigated on SNU-C4 human colon cancer cells and hesperidin-induced apoptosis was triggered by a decrease in Bcl-2, an increase in Bax and caspase-3 activation (Park, et al. 2008). Hesperidin has been reported to induce apoptosis in Panc-28 human pancreatic cells through p53-mediated intrinsic apoptosis pathway (Patil, et al. 2009). Moreover, the proliferation of Ramos Burkitt's lymphoma cells treated with hesperidin was inhibited and they became sensitive to doxorubicin-induced apoptosis through inhibition of NF- κ B (Nazari, et al. 2011). The antiproliferative and apoptotic effects of hesperidin on human pre-B NALM-6 cells were mediated via p53 accumulation and downregulation of constitutive NF- κ B activity (Ghorbani, et al. 2012). Invasion of human HepG2 human hepatocellular carcinoma cells was found to be suppressed by inhibiting NF- κ B activity, which was resulted in the inhibition of MMP-9 expression (Lee, et al. 2010).

Inhibition of cell proliferation after hesperetin treatment was found to be related to significant cell cycle arrest in the G1 phase by downregulating Cdk-2 and -4 together with cyclin D in MCF-7 breast cancer cells (Choi 2007). In another study, hesperetin was shown to reduce cell viability and induce apoptosis in SiHa human cervical cancer cell lines. (Alshatwi, et al. 2013). Hesperetin induced apoptosis by increasing expression of caspase-3, caspase-8, caspase-9, p53, Bax, and Fas death receptor. Hesperetin also induced apoptosis on HT-29 colon cancer cells by causing increases in cytochrome c, Bax and cleaved caspase-3 expression and a decrease in Bcl-2 expression (Sivagami, et al. 2012). Hesperetin showed cytotoxic activity and could activate caspase-3 and downregulate anti-apoptotic protein, Mcl-1 in HL60 cells (Chen, et al. 2003).

1.10. Vitexin

Vitexin, natural derivative of apigenin (Apigenin 8-C-glucoside), is a naturally-occurring flavonoid and lignan compound that is identified in various plant sources such as hawthorn herb (Figure 1.10). Vitexin is demonstrated to have anti-oxidative, anti-inflammatory, anti-metastatic and antitumor properties.

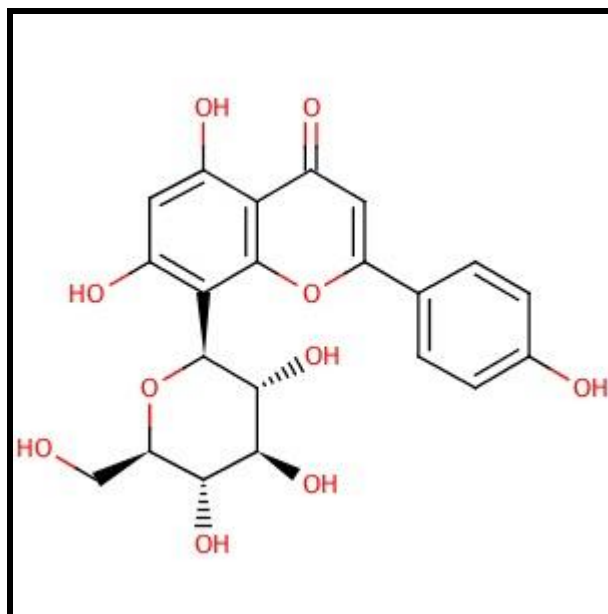


Figure 1.10. The chemical structure of vitexin (Source: Santa Cruz Biotechnology, Inc.)

1.10.1. Vitexin and Cancer: Literature Overview

In human OC2 oral cancer cells, vitexin was shown to decrease cell viability and induced apoptosis by upregulating p53 and its downstream genes p21^{WAF1} and Bax (Yang, et al. 2013). Vitexin also blocked metastatic activity in these cells, which was characterized by increased plasminogen activator inhibitor 1 (PAI-1) accumulation and decreased matrix metalloproteinase-2 expression. The effects of vitexin on human U937 leukemia cells were found to be related with the induction of apoptosis via mitochondrial pathway. Bcl-2 downregulation and upregulation of caspase-3 and caspase-9 were detected in vitexin-treated cells (Lee, et al. 2012). In neuroblastomas, vitexin acts by inducing caspase-dependent, p53-mediated apoptosis (Aggarwal, et al. 2012). In one report, vitexin was found to activate caspases, induce apoptosis, and have broad antitumor activity in breast cancer xenograft models (Zhou, et al. 2009). Vitexin compound was found to inhibit cell proliferation, induce apoptosis, and inhibit the mTOR signaling in the human choriocarcinoma JEG-3 cell line (Tan, et al. 2012). Purified vitexin 1 compound induced apoptosis HepG2 hepatocellular carcinoma cells by inhibiting phosphorylation of AKT and ERK1/2 and activating FOXO3a transcription factor (Wang, et al. 2014). Vitexin compound 1 treatment resulted in

apoptosis in MDA-MB-231 breast cancer cells, which was associated with the production of reaction oxygen species and changes in Bcl-2 family members (Liu, et al. 2014).

1.11. Aim of the Study

Leukemias are characterized by their heterogenic nature and differences in their biological characteristics and development of resistance in most cases leads to unique therapeutic problems. Even though several treatment strategies are available for CML and APL as described, they remain incurable and the emergence of drug resistance is common. Thus, novel treatment approaches are needed to overcome difficulties.

Flavonoids are known to block the initiation, promotion and progression of cancer by modulating various signaling pathways that have significant roles in cell proliferation, differentiation, apoptosis, angiogenesis and metastasis. Thus, flavonoids (both natural and synthetic analogs) are being investigated for their potentials in cancer therapy.

The purpose of this thesis study is to investigate therapeutic potentials of three plant-derived natural flavonoids, fisetin, vitexin and hesperetin, on human K562 CML and HL60 APL cells. Although these three flavonoids have been studied on several types of cancer as reviewed in the related parts, there are no studies on K562 CML cells and HL60 APL cells. The effects of these three compounds on CML and APL cells have been evaluated in terms of cytotoxicity, apoptosis and cell cycle progression. In this study, genome-wide microarray analysis has been also performed for CML and APL cells to identify the genes and networks that are responsible for fisetin and hesperetin-induced effects. Vitexin was not included for genome-wide microarray analysis since it is not effective as much as fisetin and hesperetin for both CML and APL cells. As a summary, we intended to explain the molecular mechanisms and global gene expression patterns related with the effects of these flavonoids for both CML and APL for the first time.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Vitexin (purity $\geq 97\%$), Hesperetin (purity $\geq 95\%$) and Fisetin (purity $\geq 98\%$) were obtained from Santa Cruz Biotechnology, Inc (Heidelberg, Germany). 10 mM stock solutions of these agents were prepared in DMSO and stored at -20°C . The final concentration of DMSO did not exceed more than 0.1% in culture. Penicillin-streptomycin, RPMI 1640, and fetal bovine serum were obtained from Invitrogen (Paisley, UK). Total RNA isolation kit was obtained from Macherey-Nagel (Germany). Bradford dye, and Coomassie blue, bovine serum albumine (BSA), trypan blue solution, β -mercaptoethanol, dimethylsulfoxide (DMSO) and MTT dye (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) were obtained from Sigma (USA).

2.2. Cell lines, Culture Conditions and Maintenance

Human K562 CML cells were obtained from German Collection of Microorganisms and Cell Cultures (Germany). HL60 APL cells were kindly provided by Dr. Ali Ugur Ural from Hematology Department of Gulhane Medical School. Both cells were grown and maintained in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in 5% CO_2 . Growth medium was replaced every two days. The cells were collected by centrifugation at 800 rpm for 5 minutes. The resulting pellet was dissolved in growth medium, which was inoculated into a sterile 75 cm^2 -tissue culture flask.

2.3. Measurement of Cell Growth by MTT assay

Antiproliferative effects of fisetin, vitexin and hesperetin on both K562 and HL60 cells were determined by the MTT cell proliferation assay. MTT dye

(Sigma,USA) is reduced to its insoluble formazan by NAD(P)H-dependent cellular oxidoreductase enzymes, giving a purple color in living cells. Briefly, 1×10^4 cells in 100 μ l growth medium were seeded into each well of 96-well plates. Then, the cells were treated with increasing concentrations of fisetin, vitexin or hesperetin and then incubated at 37 °C in 5% CO₂ for 48 and 72 hours. After incubation, the cells were treated with 20 μ l MTT for 3 h. Then, plates were centrifuged for 10 minutes at 580 g. After centrifugation, supernatants were removed from the plates and then the MTT crystals were homogenized by adding 100 μ l DMSO to each well under shaking. Afterwards, the plates were read at 570 nm wavelength with an ELISA reader (Thermo Electron Corporation Multiskan Spectrum, Finland). Finally, the IC₅₀ value (drug concentration inhibits cell growth by 50%) of these agents was calculated from cell proliferation plots.

2.4. Analysis of Apoptotic Cells by AnnexinV-FITC/PI Double Staining

The translocation of phosphatidylserine (PS) from the inner membrane to the outer cell membrane was measured to examine apoptotic effects of fisetin, vitexin or hesperetin on CML and APL cells. PS located on the cell surface can be easily analyzed by using a fluorescent conjugate of Annexin V (Annexin V-FITC Apoptosis Detection Kit, BioVision Research Products, USA). Initially, 5×10^5 cells were treated with increasing concentrations of agents for 72 h. Then, the cells were washed twice with cold PBS and then homogenized with 200 μ l of 1X binding buffer. Next, 2 μ l of FITC Annexin V and 2 μ l of propidium iodide (PI) were added and the final mixture was incubated for 15 minutes at room temperature in the dark. Afterwards, samples were analyzed by flow cytometry (BD Facsanto Flowcytometry, Belgium) within 1 h. It is possible to differentiate between apoptosis and necrosis when performing both Annexin V-FITC and PI staining. The percentage of early apoptotic and late apoptotic cells were summed up for each concentration of agents and untreated control samples. The obtained percentage for control sample was accepted as 1 and fold changes were calculated for each concentration as compared to control samples.

2.5. Analysis of the Changes in Mitochondrial Membrane Potential (MMP)

The loss of MMP in response to fisetin, vitexin or hesperetin in K562 and HL60 cells was detected by JC-1 Mitochondrial Membrane Potential Detection Kit (Cayman Chemicals, USA). This kit contains a unique cationic dye, JC-1, in order to determine the loss of MMP. JC-1 has the ability to enter into mitochondria and change its color from green to red reversibly. In healthy cells with high MMP, JC-1 spontaneously forms complexes in mitochondria and gives intense red fluorescence. On the other hand, in apoptotic cells with low MMP, JC-1 remains in the monomeric form and gives green fluorescence. Briefly, 5×10^5 cells in 2 mL growth medium were treated with agents for 72h and then collected by centrifugation at 180 g for 10 min. The pellets were homogenized with 300 μ l medium. Then 30 μ l of JC-1 dye was added to the cells and the cells were incubated at 37°C in 5% CO₂ for 30 min. Then, they were centrifuged at 400 g for 5 min and supernatants were removed. Then, 200 μ l assay buffer were added to the pellets, and vortexed. Then, this step was repeated. Afterwards, all pellets were homogenized with 320 μ l assay buffer and 100 μ l from each sample was added to a 96-well plate as triplicates. In healthy cells, the aggregate red form has absorption/emission maxima of 560/595 nm, whereas in apoptotic cells, the monomeric green form has absorption/emission maxima of 485/535 nm. The plate was read in these wavelengths by fluorescence Elisa reader (Thermo Varioskan Spectrum, Finland). The ratio of fluorescent intensity of JC-1 monomers (green) to fluorescent intensity of JC-1 aggregates (red) was calculated for each concentration as well as untreated control sample. The obtained ratio for control sample was accepted as 1. Then, fold changes in cytoplasmic/mitochondrial JC-1 were determined by comparing to control samples.

2.6. Analysis of Caspase-3 Activity

Changes in caspase-3 activity of the cells were examined by caspase-3 colorimetric assay kit (BioVision Research Products, USA). This assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) released from the labeled substrate DEVD-*p*NA after cleavage by caspases. Briefly, the cells (5×10^5 cells/2 mL), induced to undergo apoptosis by fisetin, hesperetin or vitexin were

collected by centrifugation at 180 g for 10 min. Then, 50 μ l of chilled cell lysis buffer was added in order to break up the cells and then incubated on ice for 10 min. Following centrifugation at 10000 g for 1 min, supernatants were collected into new tubes. The reaction cocktail including 50 μ l of 2X Reaction Buffer (containing 10 mM DTT), 50 μ l of sample, and 5 μ l of DEVD-pNA substrate was prepared and then added to 96 well plates, which was incubated for 2 h at 37 °C. Finally, the plate was read under 405 nm wavelengths by Elisa reader. Total protein concentrations determined by bradford method were used to normalize the absorbance values. The obtained value for control sample was accepted as 1. Then, fold changes in caspase-3 enzyme activities were determined by comparing to control samples.

2.7. Cell Cycle Analysis

This technique is based on the determination of amounts of dsDNA by staining with propidium iodide by flow cytometry. When the data obtained from the flow cytometry are analyzed, cell cycle phases can be determined. Briefly, 5×10^5 cells/2mL were treated with increasing concentrations of fisetin, hesperetin or vitexin for 72 h. Then, the cells were collected by centrifugation at 260 g for 10 min. Supernatants were removed, and pellets were homogenized with 1 mL cold PBS, and then the samples were put on ice. Afterwards, while the cells were slightly vortexed, 4 mL of cold ethanol was added to these cells, and then put on ice. Cells fixed by this method were incubated overnight at -20 °C for the analysis. Next day, the cells were centrifuged at 260 g for 10 min, and supernatants were completely removed from the pellets. Pellets were homogenized with 1 mL cold PBS, and centrifuged again at 260 g for 10 min. Afterwards, cell pellets were homogenized with 1 mL PBS containing 0.1% triton X-100, and then 100 μ l RNase A (200 μ g/mL) was added to these cells, and incubated at 37 °C for 30 min. After this incubation period, 100 μ l PI (1 mg/mL) was added to the cells. These cells were incubated at room temperature for 15 min, and then analyzed by flow cytometry.

2.8. Total RNA Isolation

2.5x10⁶ K562 and HL60 cells were seeded in 25 cm² tissue culture flask in 5 ml growth medium and treated with different concentrations of fisetin and hesperetin for 72 hours in 5% CO₂. Total RNA was extracted using a Nucleospin Total RNA isolation kit (Machery-Nagel, USA) as indicated by the manufacturer. The cells were lysed by adding 350 µl RA1 buffer and 3.5 µl β-mercaptoethanol. The mixture was transferred into NucloSpin[®] filter and centrifuged at 11,000 g for 1 min. Filter was removed and 350 µl ethanol (70%) was added into the solution and mixed by pipetting. The resulting mixture was transferred to NucloSpin[®] RNA Column and centrifuged at 11,000 g for 30 sec to bind RNAs to the column. Then, 350 µl membrane desalting buffer was added and centrifuged again at 11,000 g for 1 min. 95 µl of DNase reaction mixture including 10 µl reconstituted rDNase and 90 µl rDNase reaction buffer were added directly to the center of the column and incubated for 15 min at room temperature. Then, the membrane was washed three times with 200 µl RAW2, 600 µl RA3 and 250 µl RA3, respectively. RNAs were eluted by adding 60 µl of RNase-free H₂O to the center of the column, which were centrifuged at 11,000 g for 1 min. The concentrations and purities of RNA samples were measured with NanoDrop (Thermo Scientific, Wilmington, DE) spectrophotometer (260/280 nm and A260/230 nm ratios) and only the samples with an A260/A280 ratio between 1.9 and 2.1 and A260/230 ratio above 1.8 were considered for whole genome microarray analysis.

2.9. Whole Genome Microarray Analysis

Illumina Human HT-12v4 beadchip microarrays (containing ~47,000 transcripts: ~30000 genes) (Illumina, Inc., San Diego, CA) were used to assess global gene expression for each sample. To perform the sample labeling for whole genome microarray analysis (Figure 2.1), TargetAmp Nano-g Biotin-aRNA Labeling Kit for the Illumina System was used (Epicentre, Madison, USA). This experiment was performed by Done Genetics and Bioinformatics Limited Co.

2.9.1. First Strand cDNA Synthesis

The Poly (A) RNA in isolated total RNA pool (500 ng for each sample) is reverse transcribed into cDNA by using an oligo (dT)-primer containing a phage T7 RNA Polymerase promoter sequence at its 5' end. First strand cDNA synthesis is performed by SuperScript III Reverse Transcriptase (Life Technologies, Paisley, UK). Table 2.1 summarizes the ingredients included in this reaction. Briefly, The annealing of T7-Oligo (dT) primer to the RNA sample was run at 65°C for 5 minutes. Then, the mixture was put on ice for 1 min. First strand cDNA Synthesis Master Mix including first strand cDNA PreMix, DTT and SuperScript III Reverse Transcriptase (200 U/ μ l) was added to the reaction and incubated at 50°C for 30 minutes.

Table 2.1. Ingredients for First Strand cDNA synthesis

Ingredients	Amount (μ l)
RNase-free water	1
Total RNA (500 ng)	1
T7-Oligo(dT) primer	1
First strand cDNA Synthesis preMix	1.5
DTT	0.25
SuperScript III Reverse Transcriptase (200 U/ μ l)	0.25
Total volume	5

2.9.2. Second Strand cDNA Synthesis

The cDNA produced in 2.9.1 was converted to double-stranded cDNA to generate anti-sense RNA (aRNA; also called cRNA) during the subsequent *in vitro* transcription reaction. Briefly, second strand cDNA master mix including 4.5 μ l second-strand cDNA PreMix and 0.5 μ l second-strand DNA Polymerase was added to the first strand cDNA reaction mixture and incubated at 65°C for 10 minutes and then at 80°C for 3 minutes.

2.9.3. *In Vitro* Transcription of Biotin-aRNA

The *in vitro* transcription reaction produces Biotin-aRNA by incorporating Biotin-UTP into the RNA transcripts. Briefly, *in vitro* transcription master mix (Table 2.2.) was added to second strand cDNA mixture and incubated at 42°C for 4 hours in a thermocycler. Then, 2 µl of RNase-Free DNase I was added to the reaction and incubated at 37°C for 15 minutes.

Table 2.2. Ingredients for *In Vitro* Transcription Mixture

Ingredients	Amount (µl)
T7 Transcription Buffer	2
UTP/Biotin-UTP	3
NTP PreMix	10
DTT	3
T7 RNAPolymerase	2
Total volume	20

cRNA was purified to remove unincorporated NTPs, salts, and other residuals for analysis with Illumina's Direct Hybridization assay by using the Qiagen RNeasy MinElute Cleanup Kit (Qiagen). cRNA concentration was measured by NanoDrop (Thermo Scientific, Wilmington, DE) and should be 750 ng for hybridization.

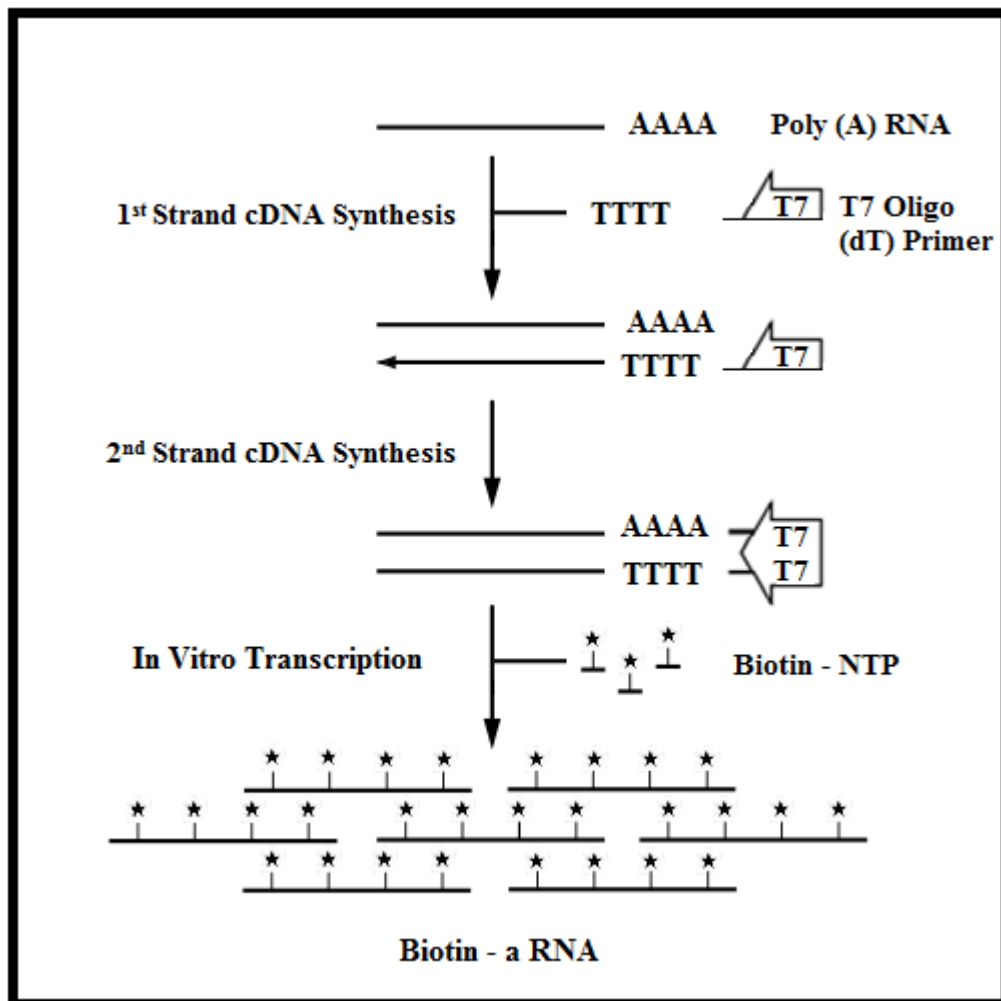


Figure 2.1. An Overview of the TargetAmp™-Nano Labeling Kit for Illumina® Expression BeadChip

2.9.4. Hybridization of BeadChip

5 µl cRNA (750 ng) was mixed with 10 µl hybridization buffer and 15 µl total mixture was loaded into sample loading channels on BeadChip surface by single-channel precision pipette. Then, hybridization was performed at 58°C for 14-20 hours in Illumina hybridization oven. The next step was washing of beadchips to remove unbound products, which were room temperature and high temperature washes in order by using specific wash buffers coming with beadchips. The final step was the labeling of BeadChips with Cy3-Streptavidin for differential detection of signals. The BeadChips were imaged using the Illumina Bead Array Reader to measure fluorescence intensity at each probe.

2.10. Statistical Analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) for MTT analyses, and 2-way ANOVA for Annexin V, MMP, and caspase-3 activity analyses. $P < 0.05$ was considered to be significant. The signal intensities corresponding to gene expression levels were imported into text files using the Illumina Genome Studio. Hierarchical cluster analysis was applied to data sets to evaluate the “proximity” between genes and hierarchical clusters were constructed with the statistically significant ($P < 0.05$) genes. Genes were considered differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 1.5 or less than 0.66 and when the p values were less than 0.05. For each comparison, we obtained the list of differentially expressed genes constrained by p-value < 0.05 and at least 2.0 fold change. Gene ontology and pathway analyses were performed to consider biological meaning of differential expression of genes between the treated and untreated samples by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cytotoxic Effects of Fisetin, Hesperetin and Vitexin on HL60 Acute Promyelocytic Leukemia and K562 Chronic Myeloid Leukemia Cells

In order to determine the cytotoxic effects of fisetin, hesperetin or vitexin on HL60 and K562 cells, the cells were treated with increasing concentrations of the agents (1-200 μM) for 48 and 72 hours and then MTT cell viability/proliferation assay was performed. There were decreases in the viability/proliferation of the cells in a dose- and time-dependent manner. IC_{50} values of fisetin at 48 and 72 hours were calculated from cell proliferation plots and were found to be 82 and 45 μM for HL60 cells and 163 and 120 μM for K562 cells (Figure 3.1) at 48 and 72 hours, respectively. On the other hand, IC_{50} values of hesperetin were found to be 190 and 142 μM for HL60 cells, and 179 and 162 μM for K562 cells (Figure 3.2) at 48 and 72 hours, respectively. IC_{50} values of vitexin were calculated to be 145 and 106 μM for HL60 cells, and 153 and 147 μM for K562 cells, respectively (Figure 3.3).

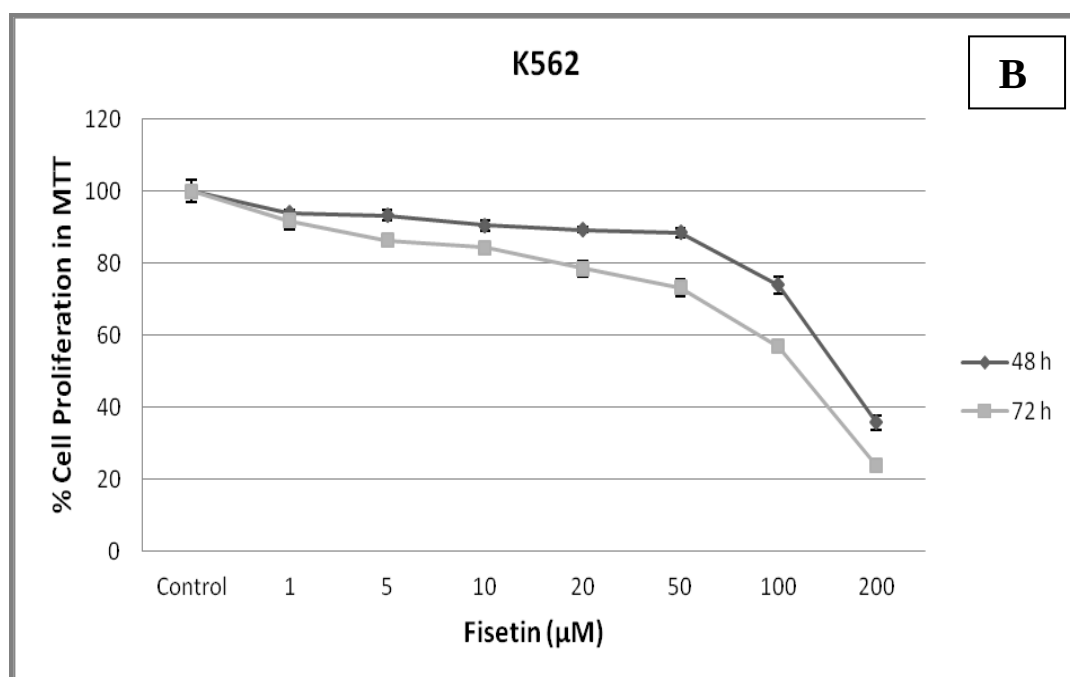
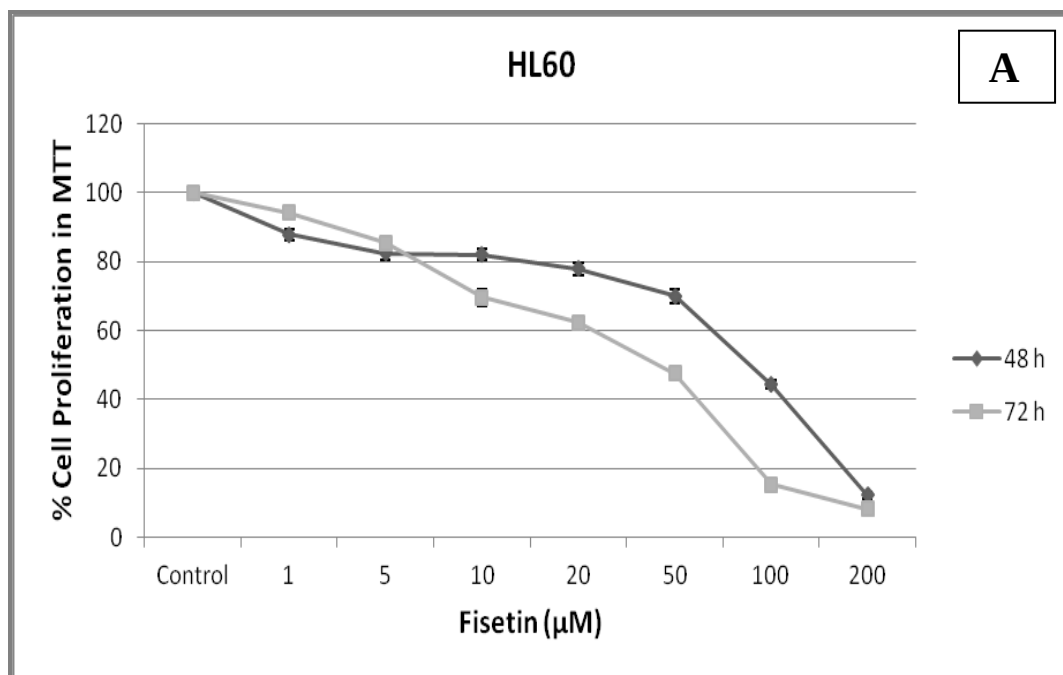


Figure 3.1. Cytotoxic effects of Fisetin on human HL60 (A) and K562 (B) cells with statistical analysis. The IC_{50} values of fitexin were calculated from cell proliferation plots. The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using one-way analysis of variance and $P < 0.05$ was considered to be significant

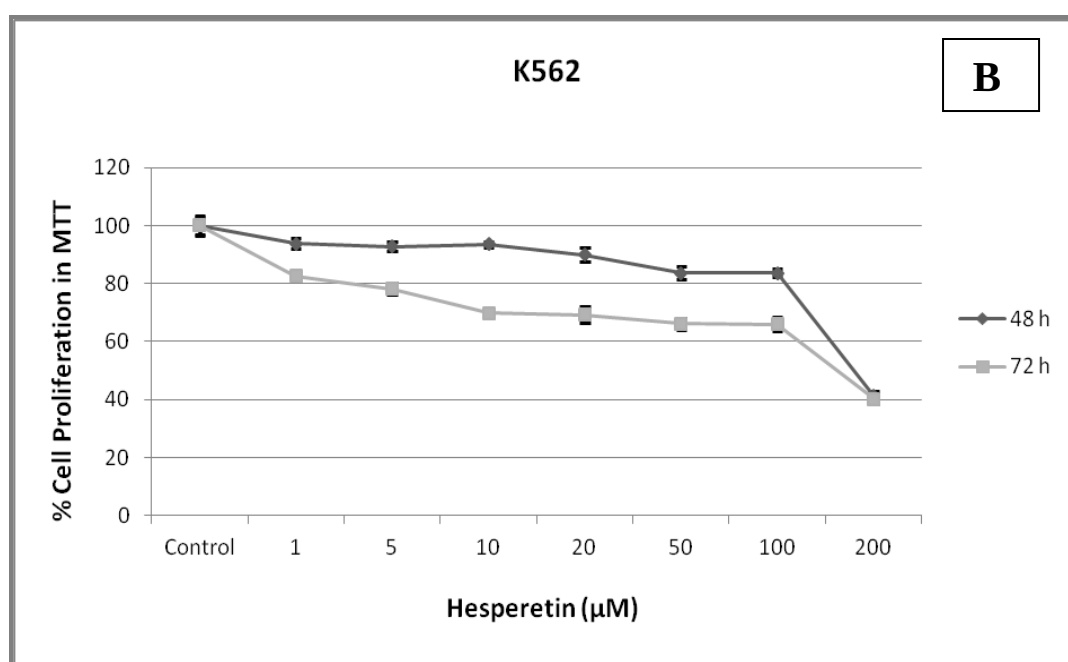
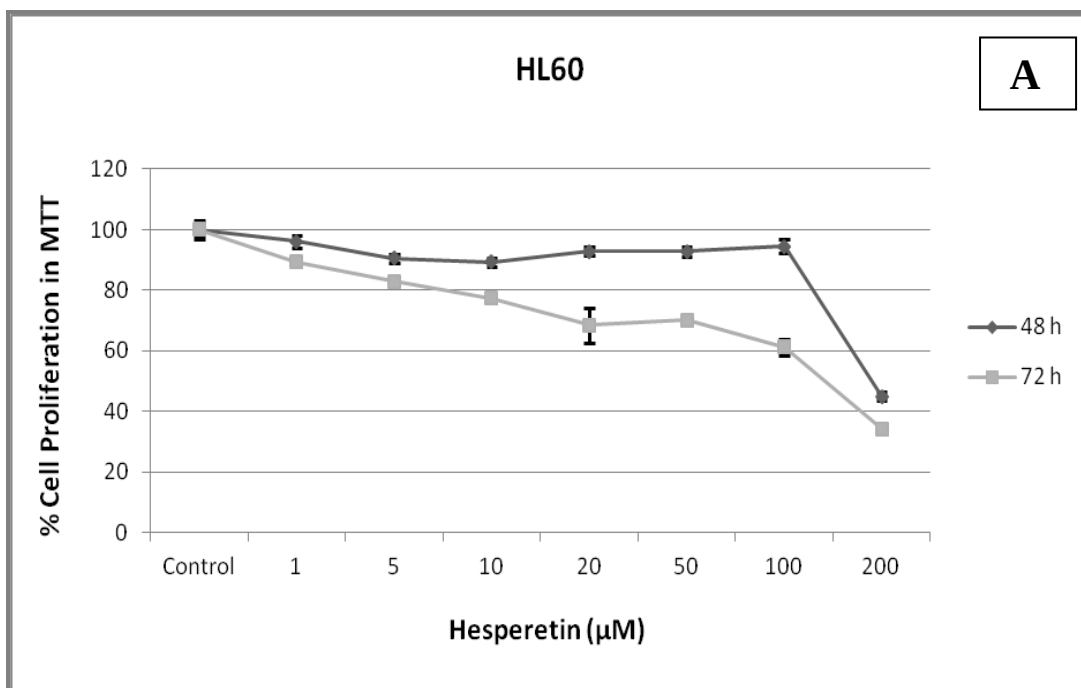


Figure 3.2. Cytotoxic effects of Hesperetin on human HL60 (A) and K562 (B) cells with statistical analysis. The IC_{50} values of hesperetin were calculated from cell proliferation plots. The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using one-way analysis of variance and $P < 0.05$ was considered to be significant

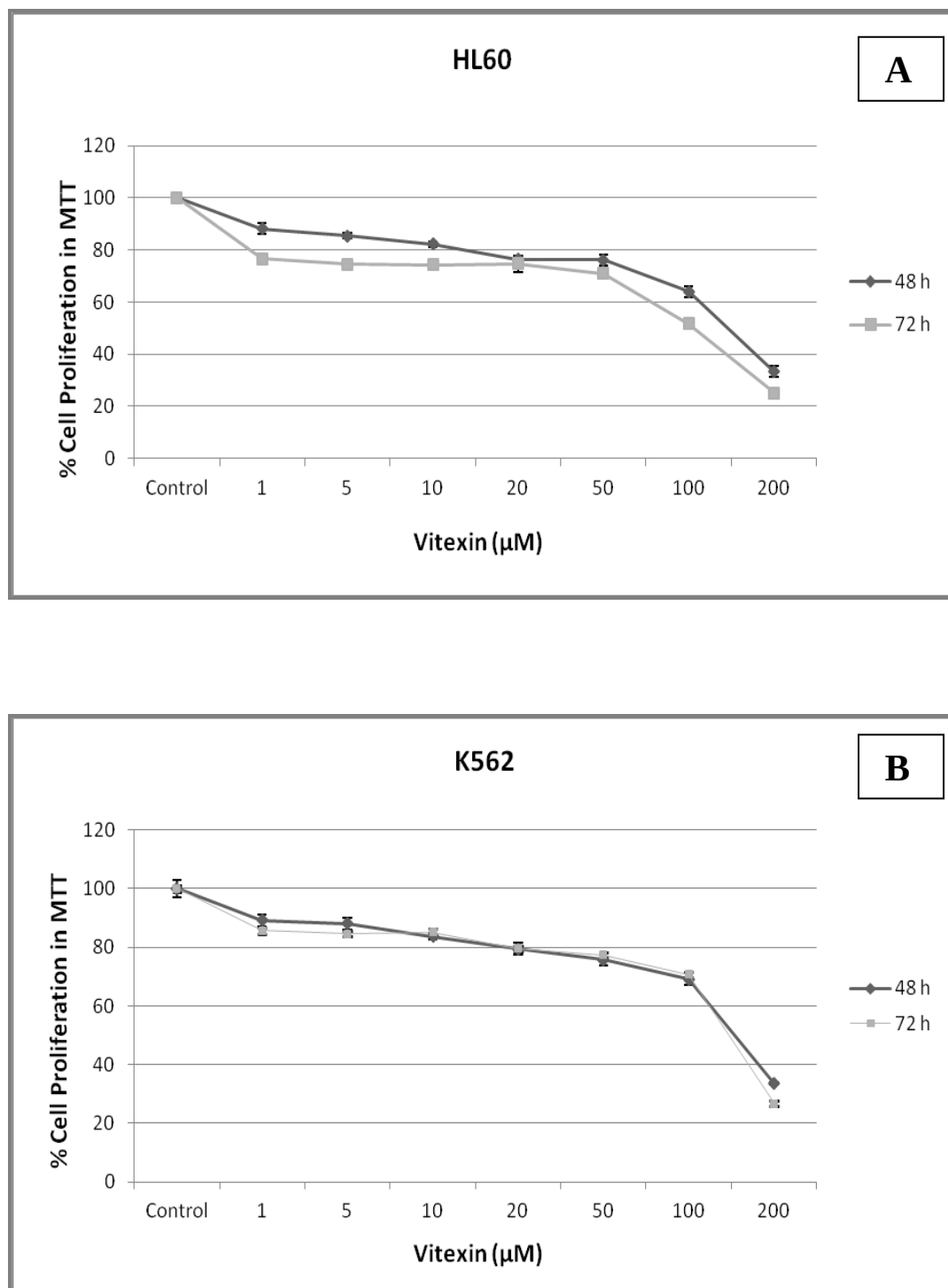


Figure 3.3. Cytotoxic effects of Vitexin on human HL60 (A) and K562 (B) cells with statistical analysis. The IC_{50} values of vitexin were calculated from cell proliferation plots. The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs Statistical significance was determined using one-way analysis of variance and $P < 0.05$ was considered to be significant

3.2. Induction of Apoptosis by Fisetin, Hesperetin and Vitexin in HL60 APL Cells

Apoptotic effects of three agents on HL60 cells were determined by annexin-V/PI double staining. The cells were incubated with increasing concentrations of the agents for 72 hours and the percentage of apoptotic cell population (late apoptotic plus early apoptotic) of the cells in response to increasing concentrations of them was determined by flow cytometry. Figure 3.4 showed the fold changes in the percentage of apoptotic HL60 cells as compared to untreated controls while Figure 3.5 indicated flow data. Fisetin treatment resulted in 1.6-, 6.2- and 11.6-fold increases in apoptosis in response to 20-, 50- and 100 μ M fisetin, respectively. On the other hand, there were 1.2-, 2.1- and 3.3- fold increases in hesperetin-treated and 1.3-, 1.5- and 2.0- fold increases in vitexin-treated HL60 cell in terms of apoptotic cell population. Fisetin was found to be the most effective flavonoid for the induction of apoptosis in HL60 cells.

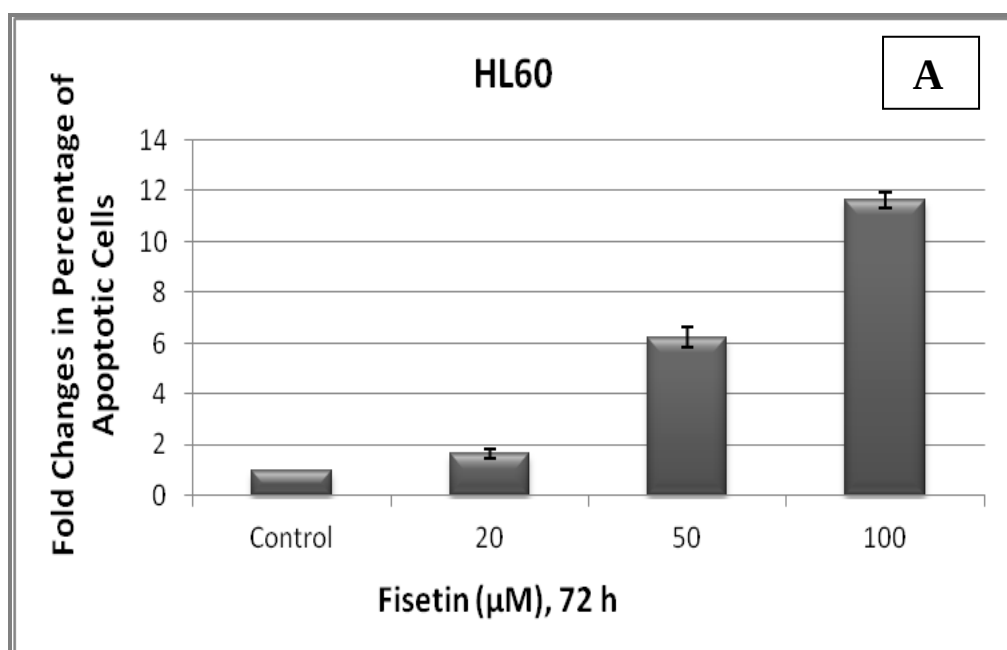


Figure 3.4. Evaluation of apoptosis in HL60 cells induced by Fisetin (A), Hesperetin (B) and Vitexin (C) by FACS analysis via Annexin V-FITC/PI staining. The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using 2-way analysis of variance and $P < 0.05$ was considered to be significant

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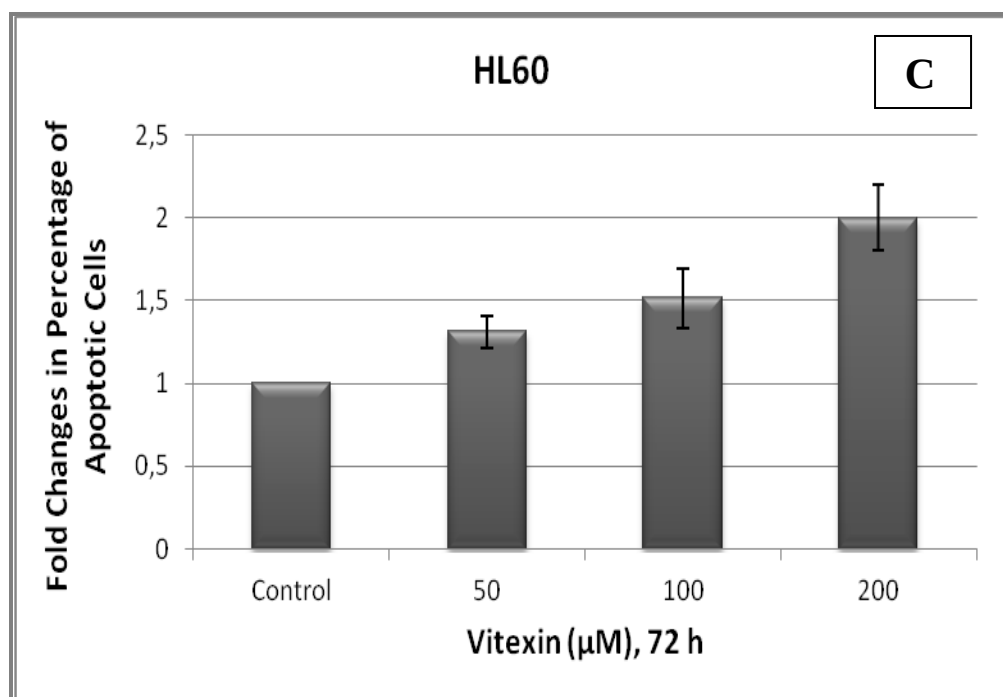
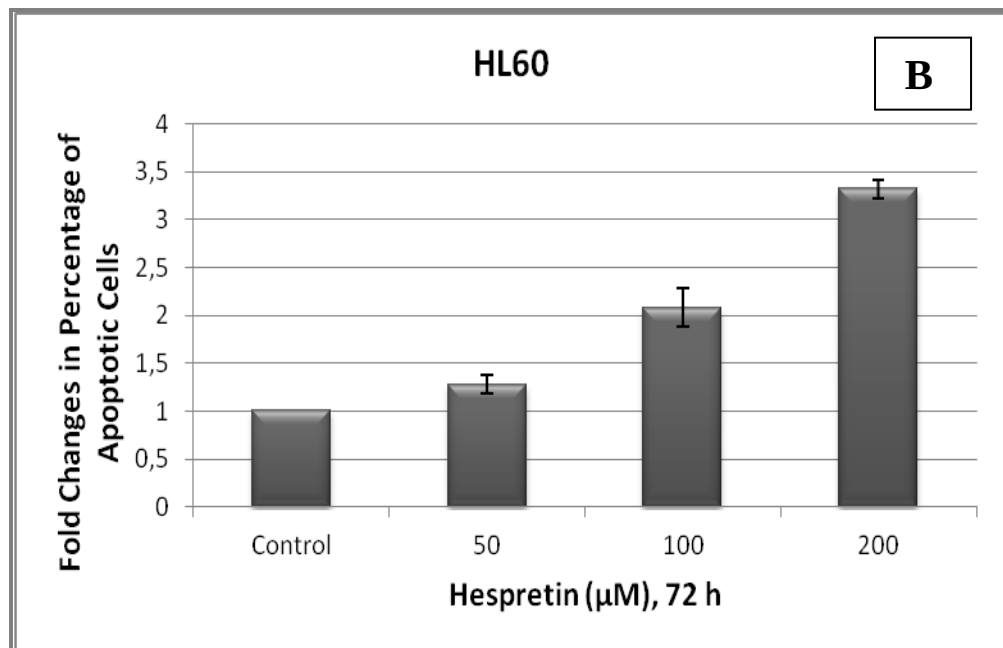


Figure 3.4 (cont.)

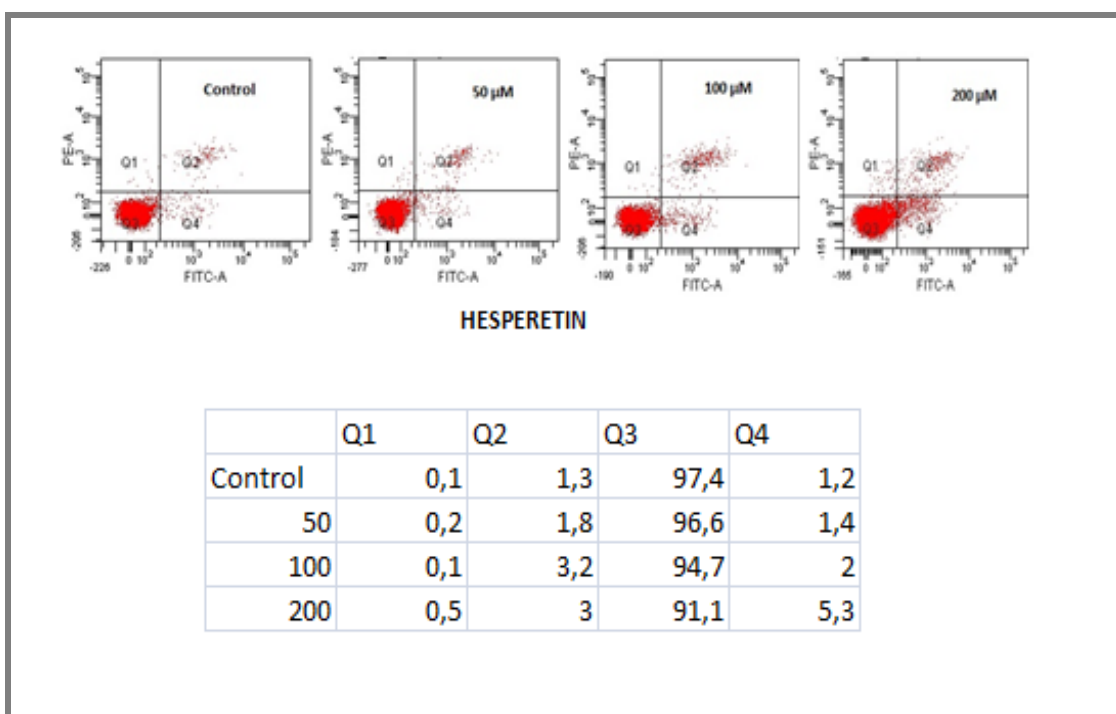
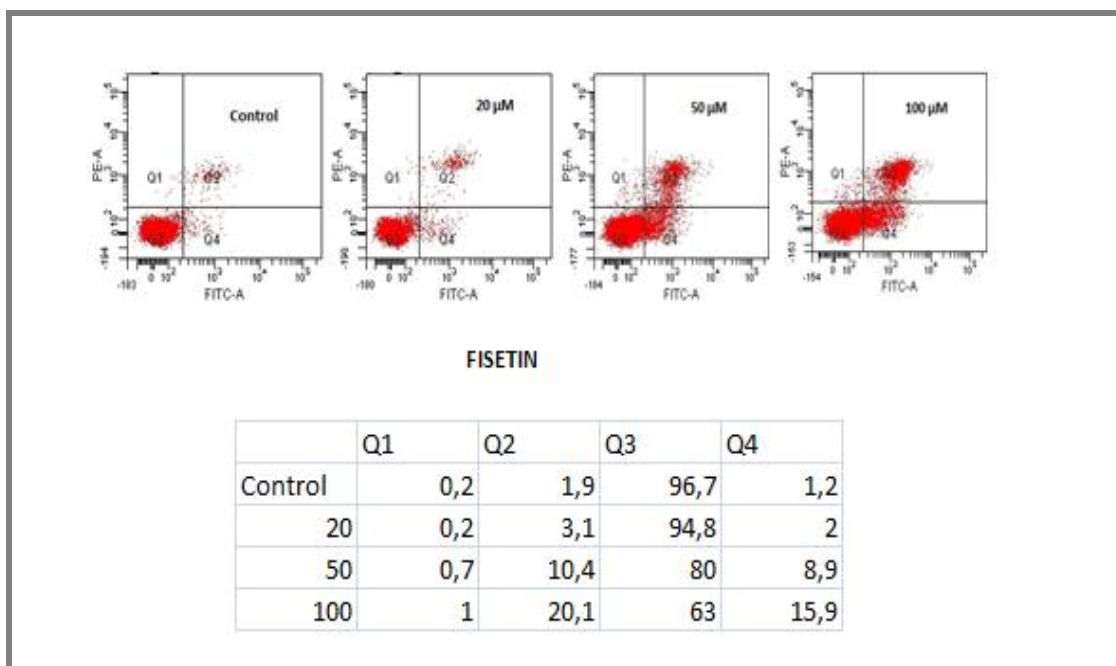


Figure 3.5. Evaluation of apoptosis in HL60 cells induced by fisetin, hesperetin and vitexin by FACS analysis via Annexin V-FITC/PI staining. Cells in the lower right quadrant indicate Annexin-positive/PI negative (Q4), early apoptotic cells. The cells in the upper right quadrant indicate Annexin-positive/PI positive, late apoptotic (Q2)

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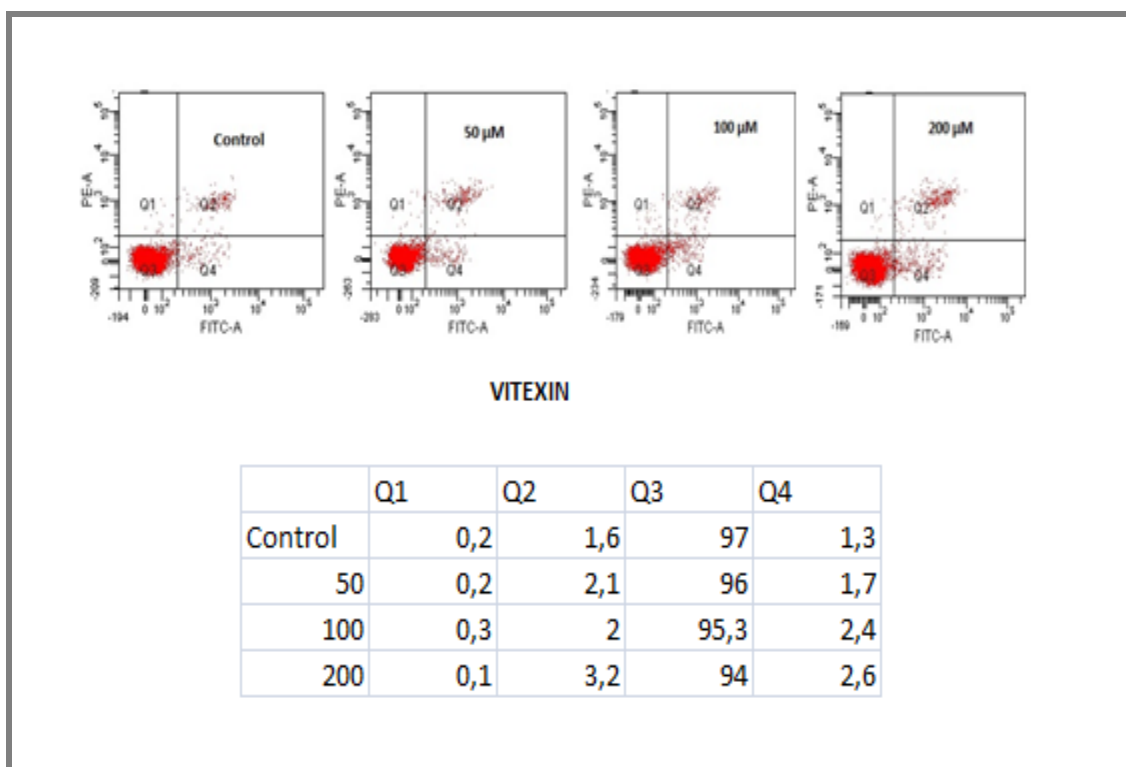


Figure 3.5 (cont.)

3.3. Induction of Apoptosis by Fisetin, Hesperetin and Vitexin in K562 CML Cells

In order to determine whether fisetin, hesperetin and vitexin induce apoptosis, the cells were incubated with increasing concentrations of the agents for 72 hours and the percentage of apoptotic cell population (late apoptotic plus early apoptotic) of the cells in response to increasing concentrations of them was determined by flow cytometry. There were 5.4-, 9.5- and 11.6 -fold increases in the percentage of apoptosis in fisetin-treated, 1.27-, 1.8- and 4.3-fold increases in hesperetin-treated and 1.1-, 1.5- and 3.6-fold increases in vitexin-treated K562 cells when compared to untreated controls as shown in Figures 3.6 and 3.7. The data showed that fisetin triggers the strongest apoptosis in a dose- dependent manner among other flavonoids similar to HL60 cells. Vitexin is the less effective agent based on these results.

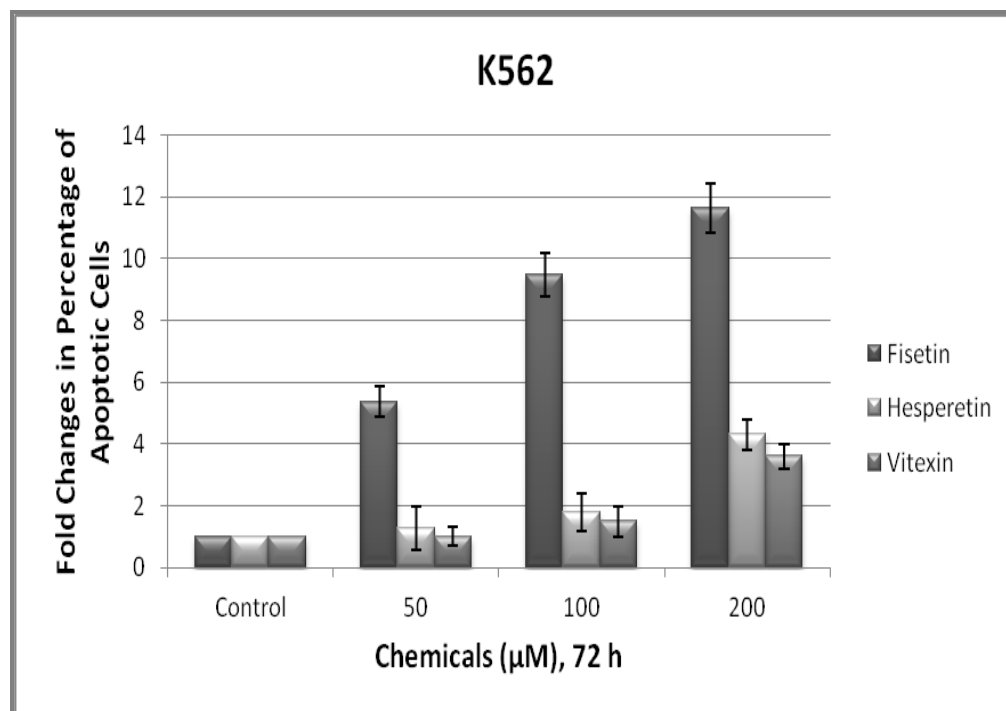


Figure 3.6. Evaluation of apoptosis in K562 cells induced by Fisetin, Hesperetin and Vitexin by FACS analysis via Annexin V-FITC/PI staining. The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using 2-way analysis of variance and $P < 0.05$ was considered to be significant

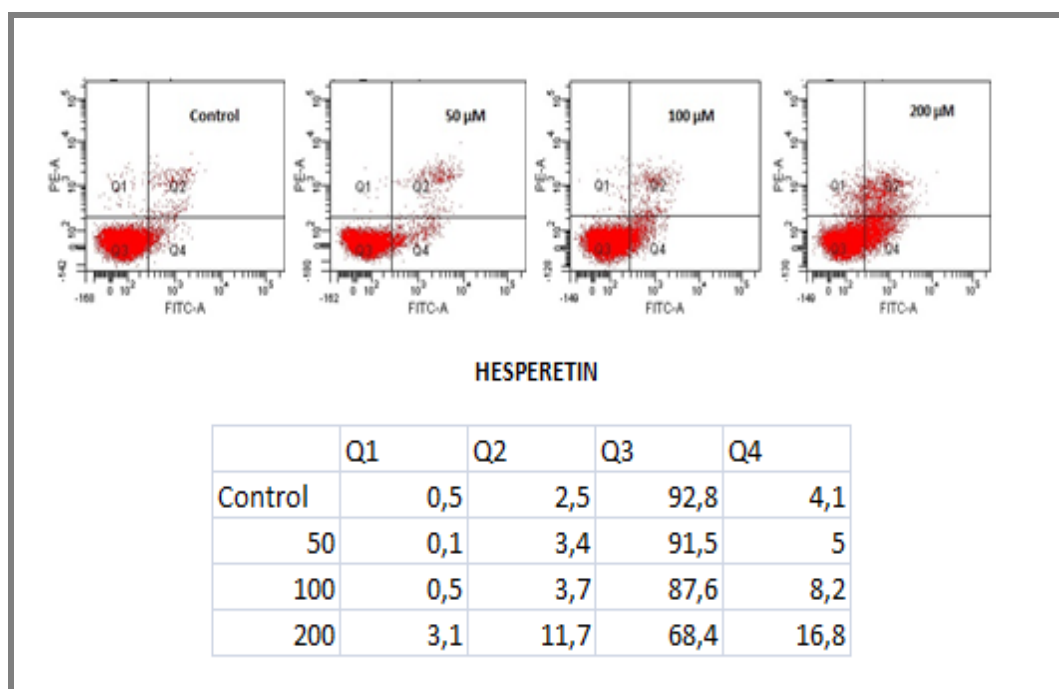
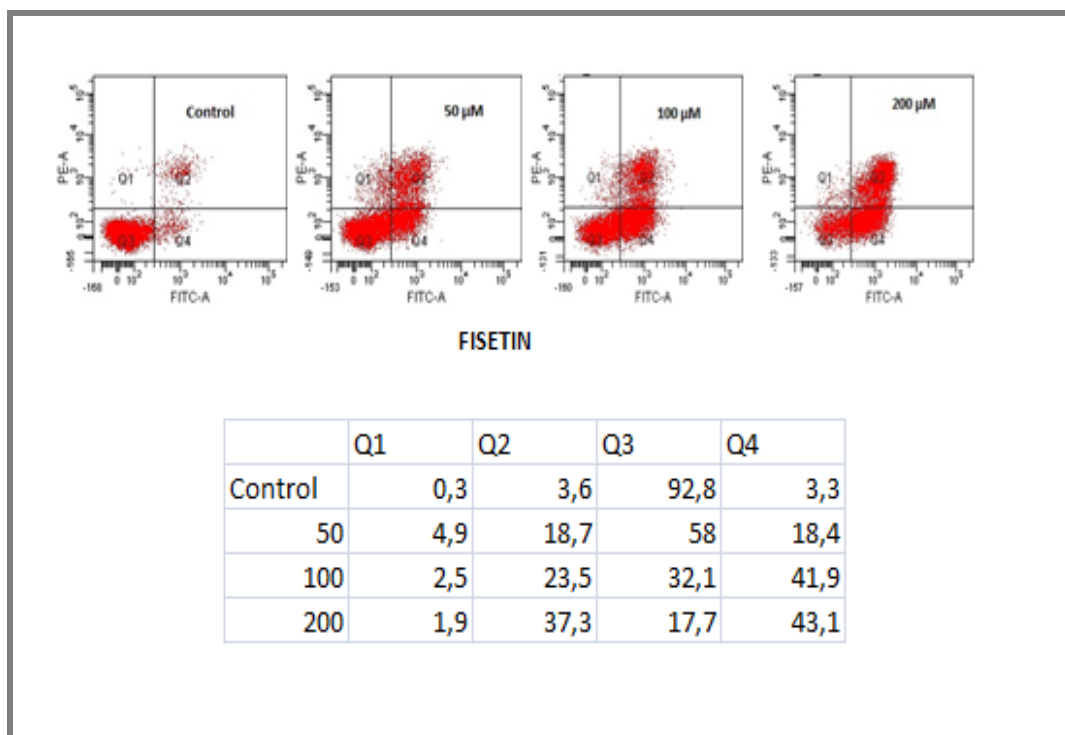


Figure 3.7. Evaluation of apoptosis in K562 cells induced by fisetin, hesperetin and vitexin by FACS analysis via Annexin V-FITC/PI staining. Cells in the lower right quadrant indicate Annexin-positive/PI negative (Q4), early apoptotic cells. The cells in the upper right quadrant indicate Annexin-positive/PI positive, late apoptotic (Q2)

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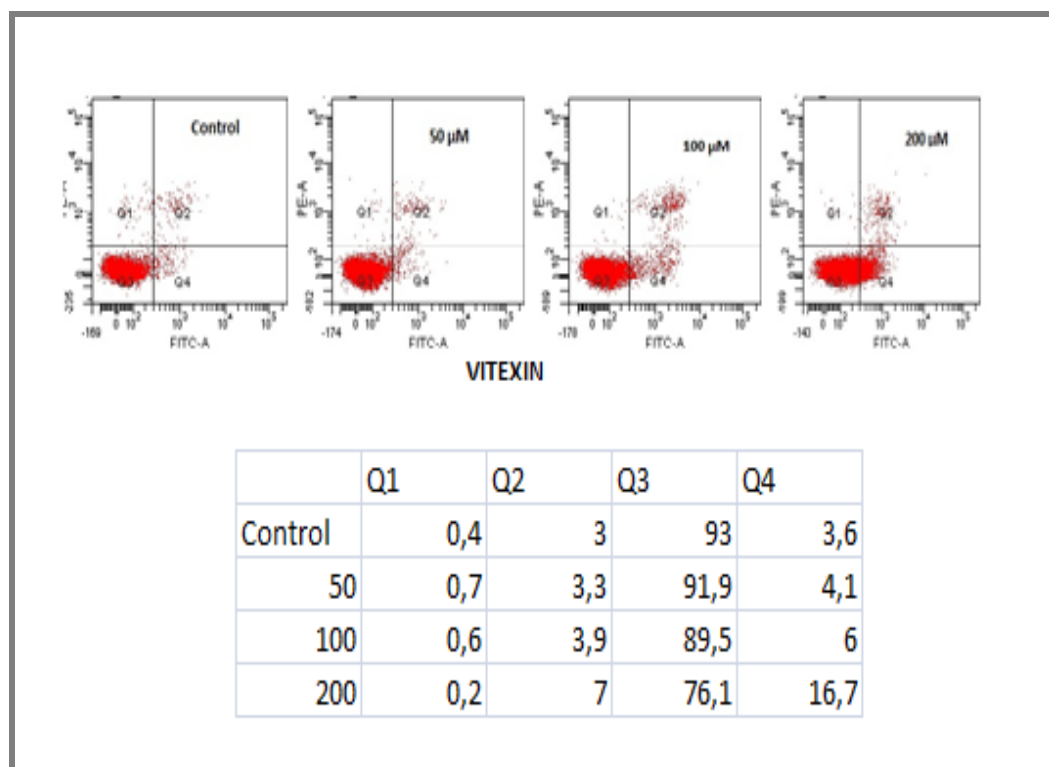


Figure 3.7 (cont.)

3.4. Evaluation of the Effects of Fisetin, Hesperetin and Vitexin on Loss of Mitochondrial Membrane Potential in HL60 Cells

Loss of mitochondrial membrane potential is an important sign of apoptosis since it has been linked to initiation and activation of apoptotic cascades. Therefore, it was determined whether these flavonoids causes the loss of MMP in APL cells exposed to increasing concentrations of them. Treatment of HL60 cells with 20-, 50- and 100 μ M fisetin caused 1.01-, 1.15- and 1.37 fold increases in loss of MMP, respectively (Figure 3.8). Figure 3.8. showed that hesperetin treatment resulted in 1.9-, 2.4- and 5.4 fold increases while vitexin caused 1.1-, 1.5- and 1.6 fold changes in loss of MMP in HL60 cells in response to 50-, 100- and 200 μ M, respectively.

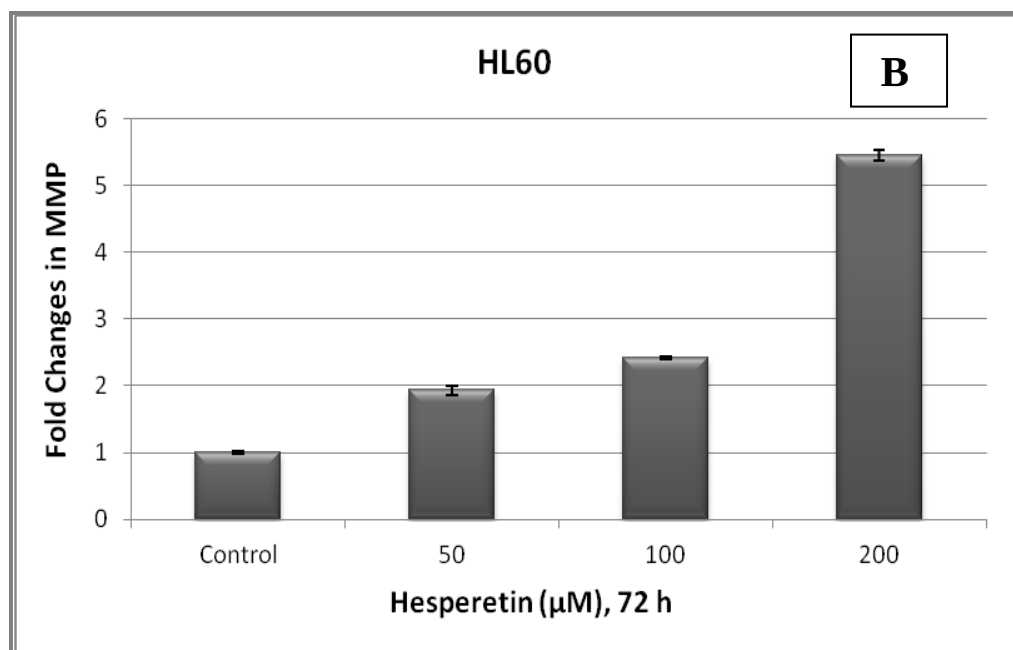
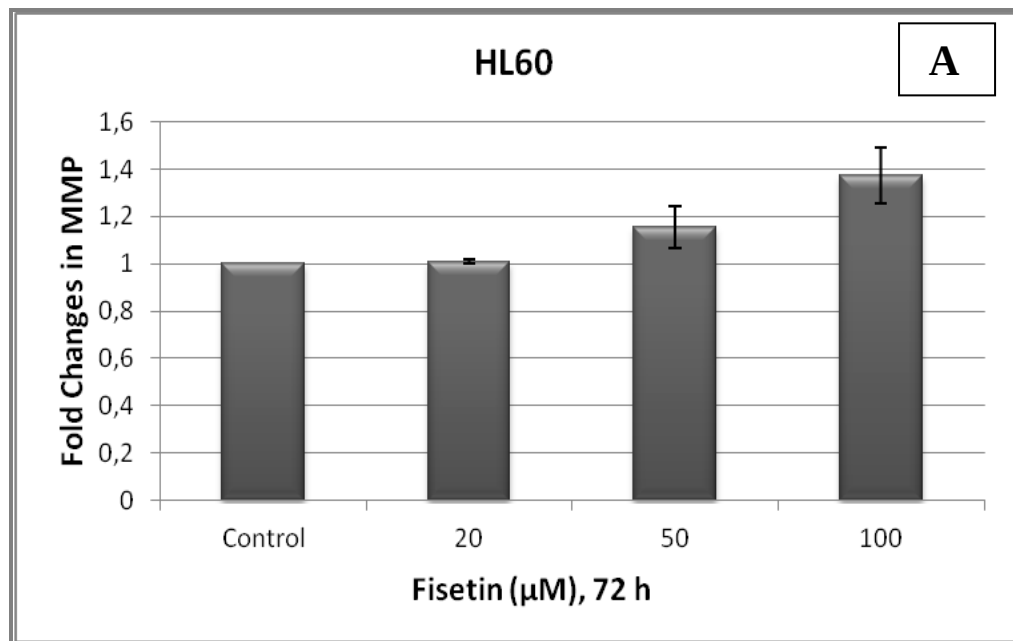


Figure 3.8. Fold changes in cytoplasmic/mitochondrial JC-1 in HL60 cells treated with increasing concentrations of Fisetin (A), Hesperetin (B) and Vitexin (C). The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using 2-way analysis of variance and $P < 0.05$ was considered to be significant

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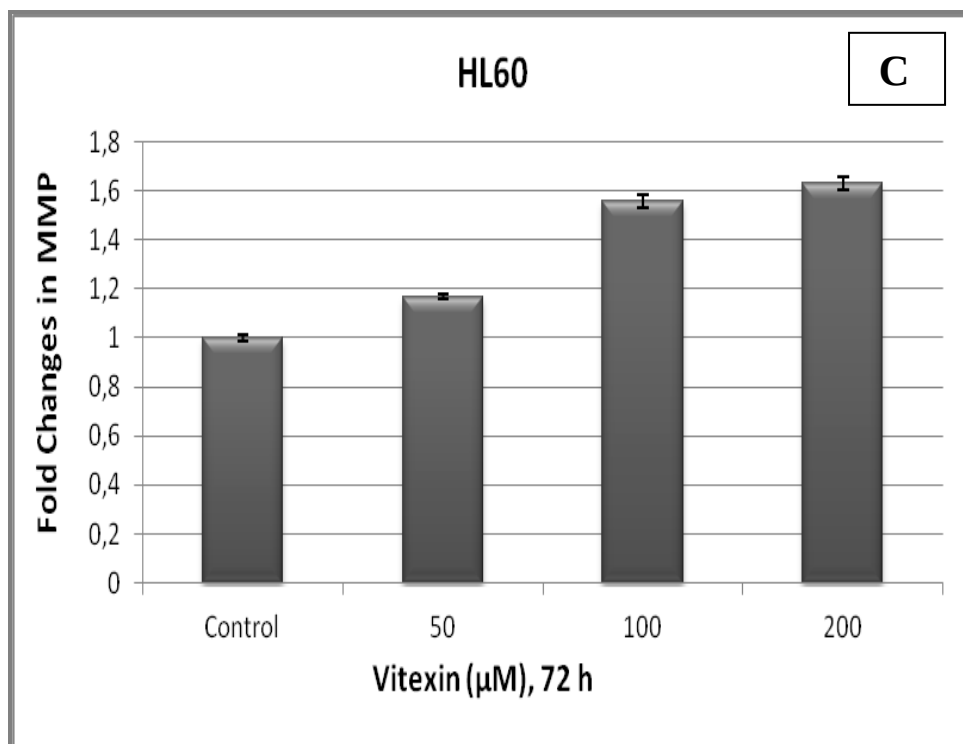


Figure 3.8 (cont.)

3.5. Evaluation of the Effects of Fisetin, Hesperetin and Vitexin on Loss of Mitochondrial Membrane Potential in K562 Cells

The effects of fisetin, hesperetin and vitexin on MMP were evaluated by using JC-1 dye based method. There were 1.03-, 1.25- and 80-fold increases in loss of MMP in K562 cells treated with 50-, 100-, and 200 μ M fisetin, respectively, as compared to untreated control cells (Figure 3.9). As shown in Figure 3.9, there were 1.7-, 2.4- and 2.5- fold increases in loss of MMP in hesperetin-treated and 2.2-, 2.6- and 2.8- fold increases in vitexin-treated K562 cells in response to 50-, 100- and 200 μ M agents, respectively.

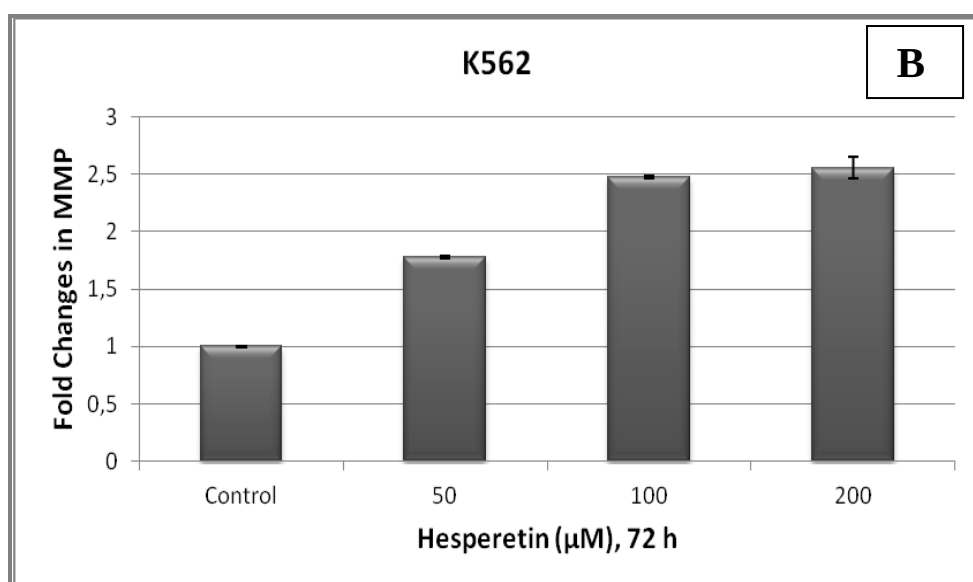
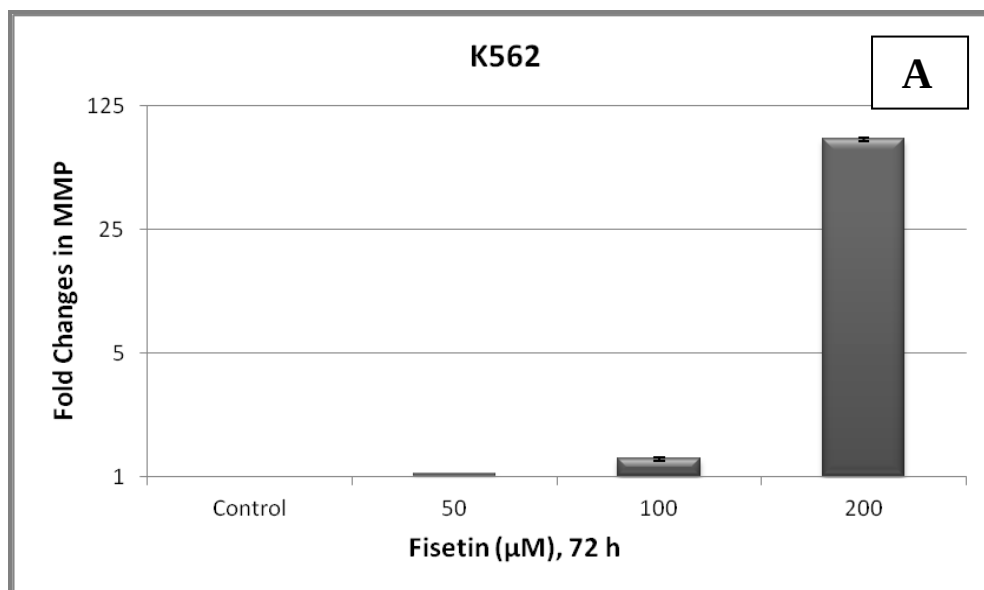


Figure 3.9. Fold changes in cytoplasmic/mitochondrial JC-1 in K562 cells treated with increasing concentrations of Fisetin (A), Hesperetin (B) and Vitexin (C). The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using 2-way analysis of variance and $P < 0.05$ was considered to be significant

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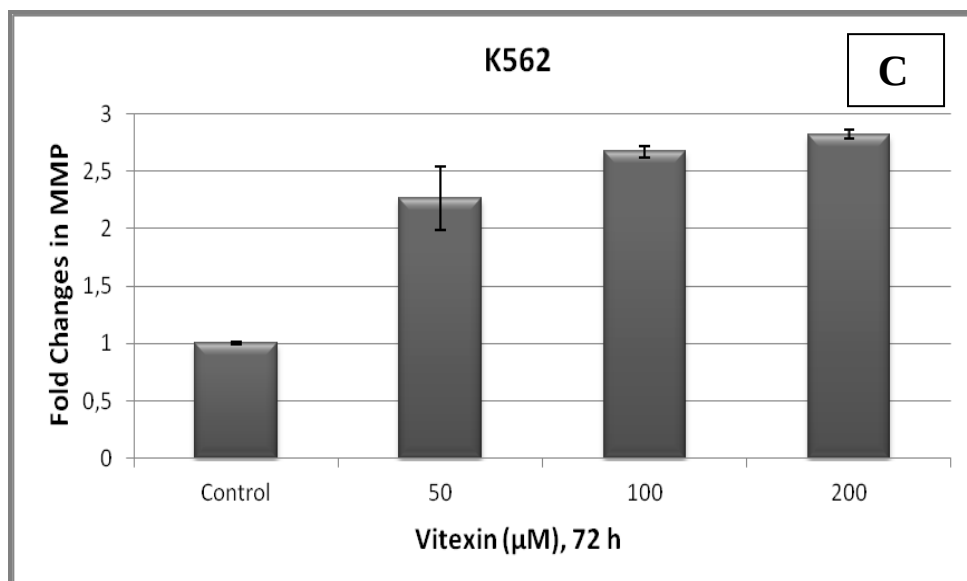


Figure 3.9 (cont.)

3.6. Evaluation of the Effects of Fisetin, Hesperetin and Vitexin on Caspase-3 Activity in HL60 Cells

Caspase-3 enzyme has a central role in both mitochondrial-mediated and death receptor related cell death. To determine whether apoptosis induced by fisetin, hesperetin or vitexin was associated with activation of caspase-3, the cells were treated with increasing concentrations of these agents for 72 h and the changes in caspase-3 enzyme activity were detected through the cleavage of the labeled substrate DEVD-pNA. As indicated in Figure 3.10., there were 1.05-, 2.0- and 2.2- fold increases in caspase-3 activity of fisetin- treated HL60 cells in response to 20-, 50- and 100 μM, respectively, as compared to untreated controls. On the other hand, there were very small changes in caspase-3 enzyme activity of HL60 cells treated with 50- (1.01-fold), 100- (1.03-fold) and 200 (1.1-fold) μM vitexin when compared to untreated controls. Treatment of HL60 cells with 50-, 100- and 200 μM hesperetin caused 1.08-, 1.1- and 2.8- fold increases in caspase-3 enzyme activity, respectively (Figure 3.10).

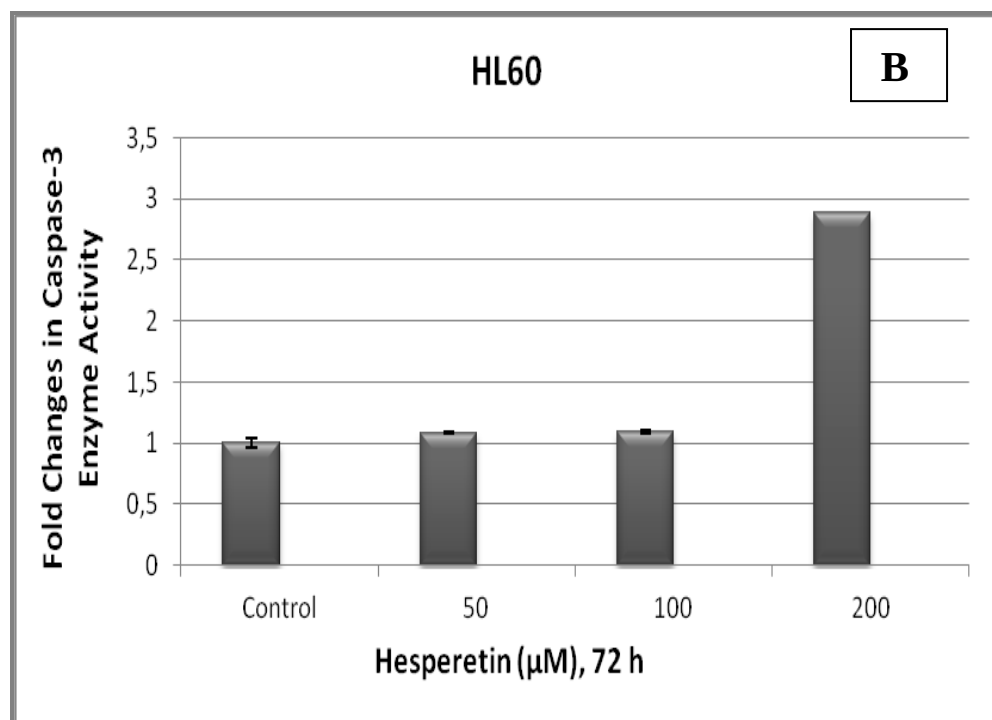
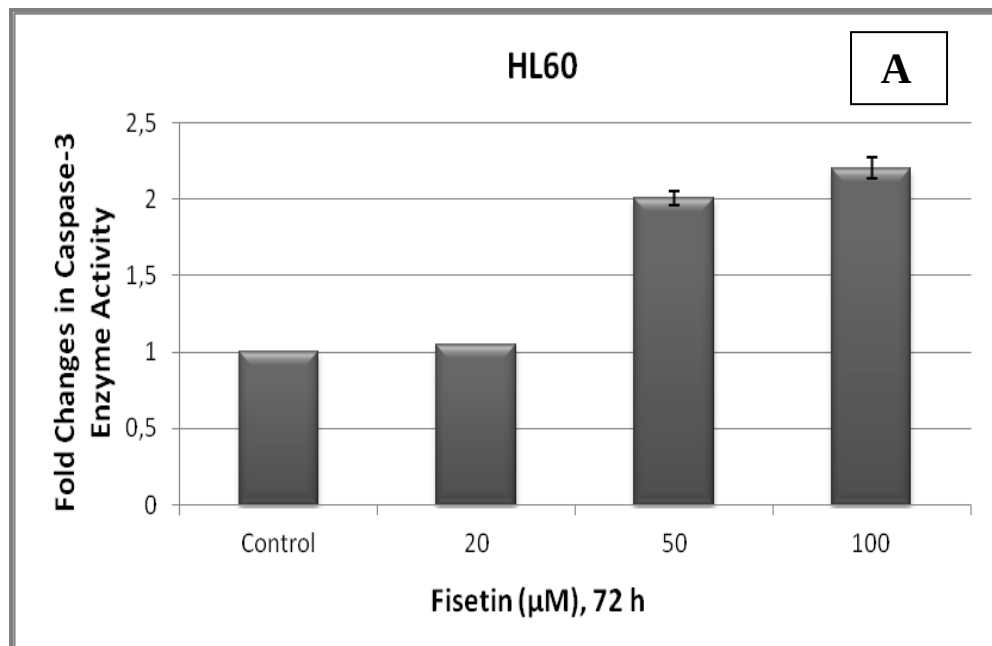


Figure 3.10. Changes in caspase-3 enzyme activity in response to Fisetin (A), Hesperetin (B) and Vitexin (C) in HL60 Cells. The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using 2-way analysis of variance and $P < 0.05$ was considered to be significant

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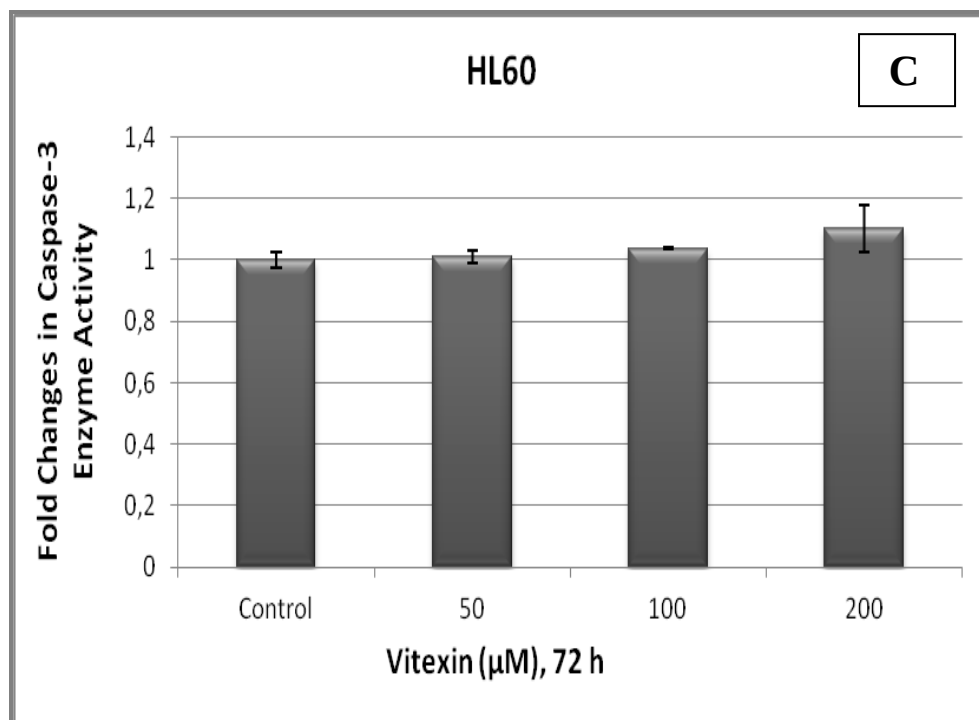


Figure 3.10 (cont.)

3.7. Evaluation of the Effects of Fisetin, Hesperetin and Vitexin on Caspase-3 Activity in K562 Cells

In fisetin-treated K562 cells, there were 1.3-, 1.6- and 1.8- fold increases in caspase-3 enzyme activity in response to 50-, 100-, and 200 μM fisetin, respectively, as compared to untreated controls. On the other hand, there were no significant changes in caspase-3 enzyme activity of K562 cells treated with 50- and 100 μM hesperetin but there was a significant (2.8-fold) increase in response to 200 μM hesperetin. Treatment of K562 cells with vitexin resulted in very small increases at 50- and 100 μM vitexin (1.01- and 1.02- fold, respectively) but there was a 1.4- fold increase at 200 μM vitexin (Figure 3.11).

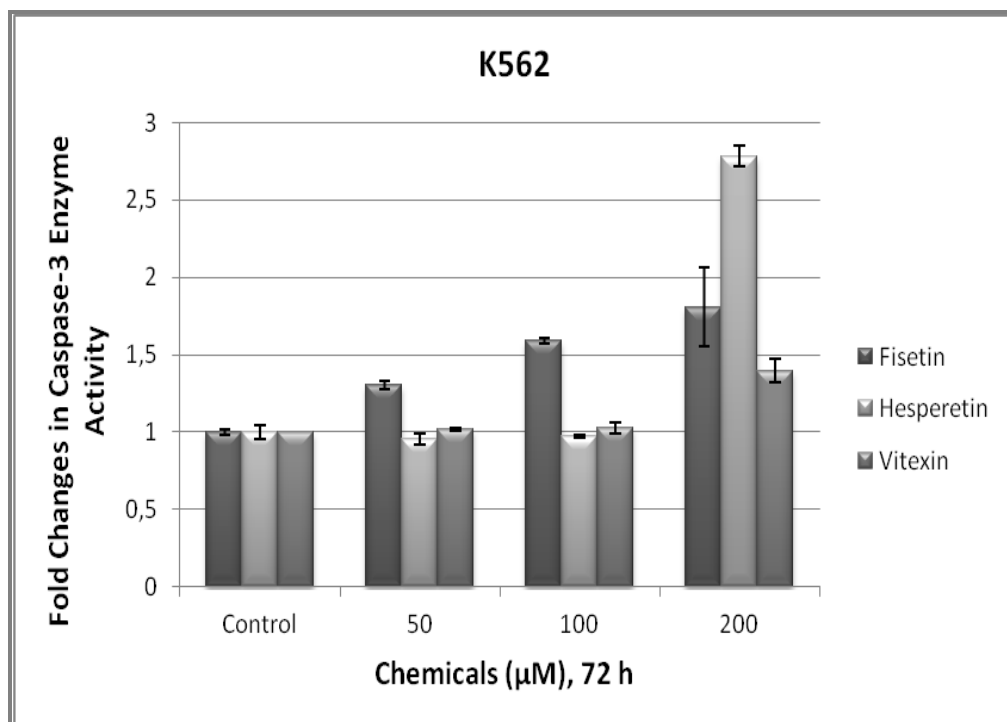


Figure 3.11. Changes in caspase-3 enzyme activity in response to Fisetin (A), Hesperetin (B) or Vitexin (C) in K562 Cells. The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using 2-way analysis of variance and $P < 0.05$ was considered to be significant.

3.8. Evaluation of the Effects of Fisetin, Hesperetin and Vitexin on Cell Cycle Progression of HL60 Cells

In order to determine the possible mechanism of antiproliferative activity of the agents, cell cycle profiles of APL cells in response to 72 hour exposure to fisetin, hesperetin and vitexin were examined by flow cytometry in the presence of DNase-free RNase and PI dye. Treatment of HL60 cells with fisetin resulted in a dose-dependent increase in the percentage of cells in the G2/M phase, which was accompanied by a reduction in the percentage of cells in the G0/G1 phase. The G2/M phase cell cycle distribution was 0.89, 8 and 29.5 % at 20, 50 and 100 μ M concentrations of fisetin, respectively (Control value: 0.57%).

On the other hand, hesperetin treatment was found to result in a dose-dependent increase of cell population in the G2/M phase of the cell cycle. The G2/M phase cell cycle distribution was 1.64, 3.65 and 13% at 50, 100 and 200 μ M concentrations of

hesperetin, respectively (Control value: 0.46%). Moreover, the percentage of the cells in the G0/G1 phase was found to increase especially at 100 (34.8%) and 200 (44.3%) μM hesperetin as compared to control (31.13%) while there was a dose-dependent decrease in the S phase population.

Vitexin treatment caused changes only at 200 μM concentration. There were increases in the G2/M and G0/G1 phases while % cell population decreased in the S phase at 200 μM concentration as compared to untreated control (Control values: 29.07% at G0/G1, 70.08% at S phase and 0.125% at G2/M phase; % values at 200 μM vitexin: 35.2% at G0/G1, 51.71% at S phase and 13.05% at G2/M phase) (Figure 3.12).

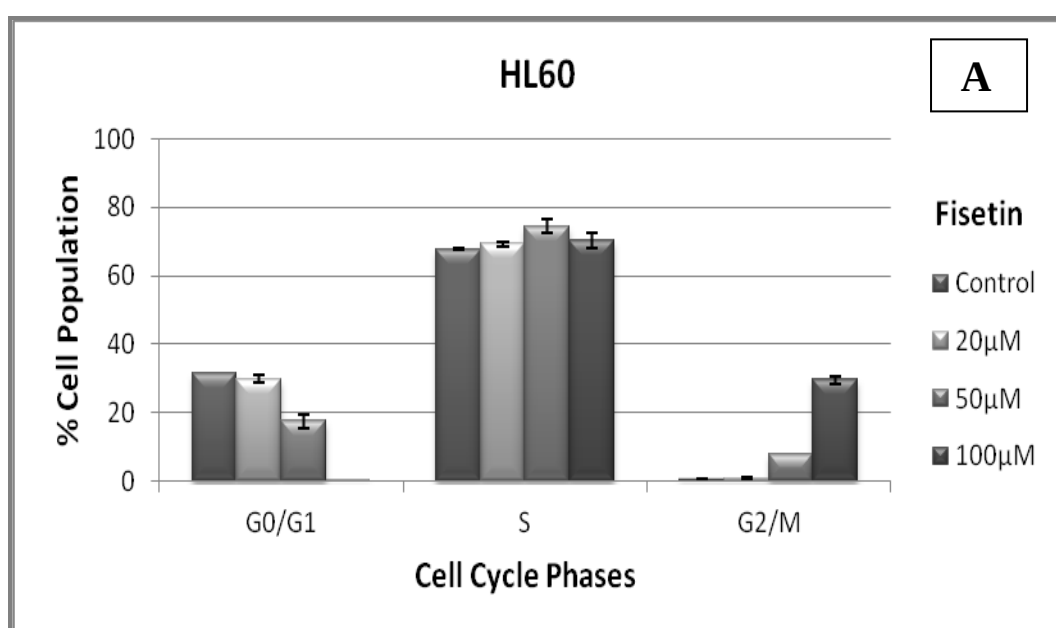


Figure 3.12. Cell cycle distribution of HL60 cells after treatment with Fisetin (A), Hesperetin (B) and Vitexin (C). The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using 2-way analysis of variance and $P < 0.05$ was considered to be significant

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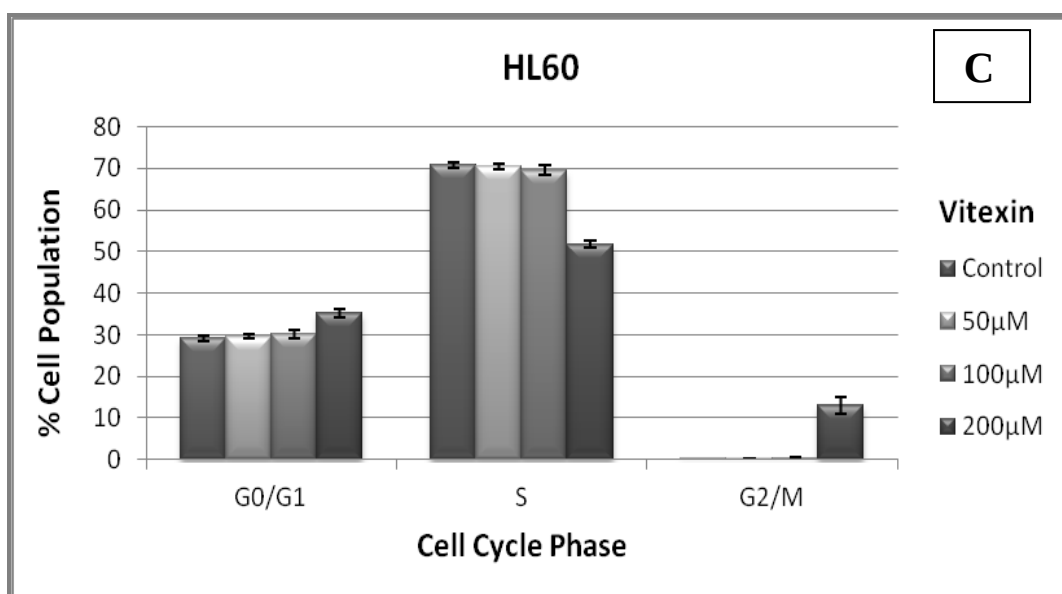
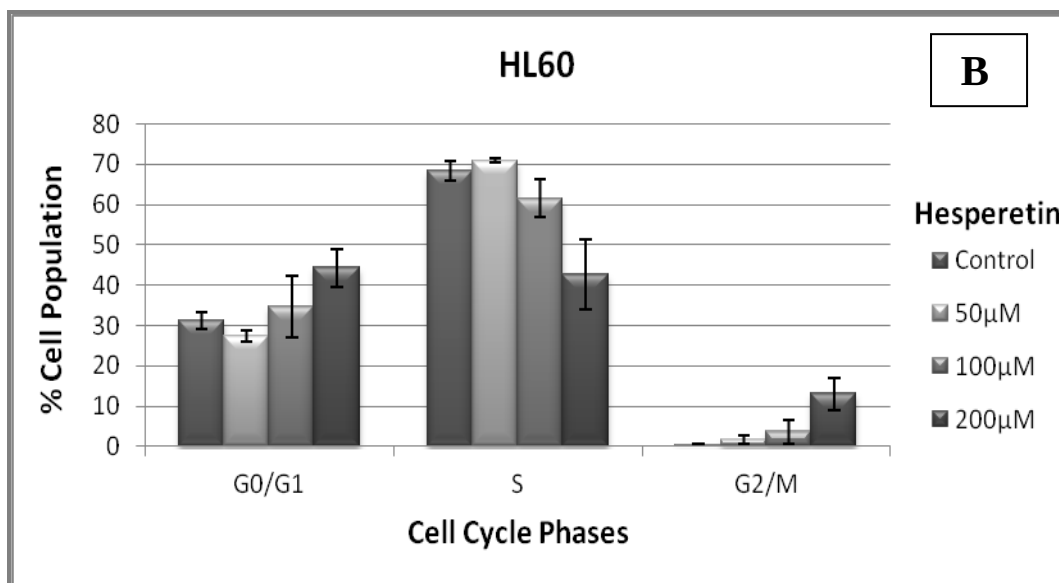


Figure 3.12 (cont.)

3.9. Evaluation of the Effects of Fisetin, Hesperetin and Vitexin on Cell Cycle Progression of K562 Cells

Figure 3.13 summarized the effects of fisetin, hesperetin and vitexin on the cell cycle progression of K562 cells. The cell cycle progression of fisetin-treated K562 cells was found to be arrested at both S and G2/M phases, which was accompanied by a

reduction in the G0/G1 phase in a dose-dependent manner. The percentage of the cells accumulated in S phase were 40.26% (Control), 48.04% (20 μ M) and 77.045% (50 μ M) while % values at G2/M phase were 2.1 (Control), 4.68 (20 μ M) and 8 (50 μ M). Furthermore, The G0/G1 phase cell cycle distribution was 57,645 (Control), 47,285 (20 μ M) and 15,955 % (50 μ M).

Hesperetin treatment resulted in a significant increase in cell population of G0/G1 phase at only 200 μ M (73.83%) concentration with respect to control (50.08%) although there were small dose-dependent increases in the G0/G1 population. Furthermore, there was a dose-dependent decrease in the S phase population (Control: 46.78%, 50 μ M: 40.49%, 100 μ M: 40.39%, 200 μ M: 22.7%).

The percentage of the cells in the G0/G1 and G2/M phases increased after vitexin treatment, which was accompanied by a reduction in the percentage of cells in the S phase (% values at G0/G1 : Control: 47.56, 50 μ M: 48.21, 100 μ M: 53.03, 200 μ M:72.21. % values at S phase: Control: 49.52, 50 μ M: 48.9, 100 μ M: 42.2, 200 μ M: 20.5. % values at G2/M phase: Control: 1.92, 50 μ M: 2.8, 100 μ M: 4.7, 200 μ M: 7.2).

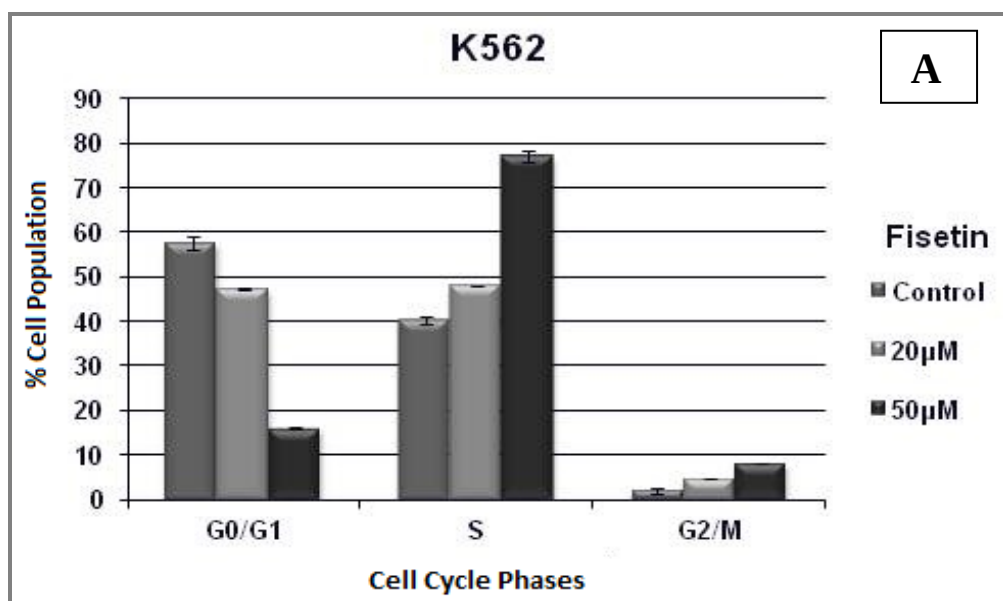


Figure 3.13. Cell cycle distrubution of K562 cells after treatment with Fisetin (A), Hesperetin (B) and Vitexin (C). The results, the means of two independent experiments, are presented as mean $\pm$ SEM, when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using 2-way analysis of variance and $P < 0.05$ was considered to be significant

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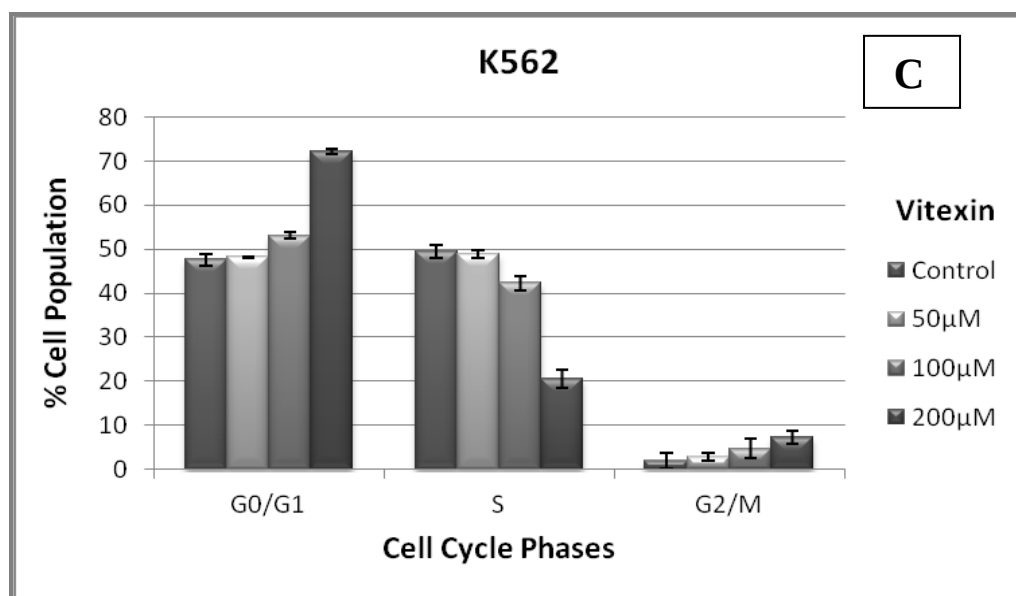
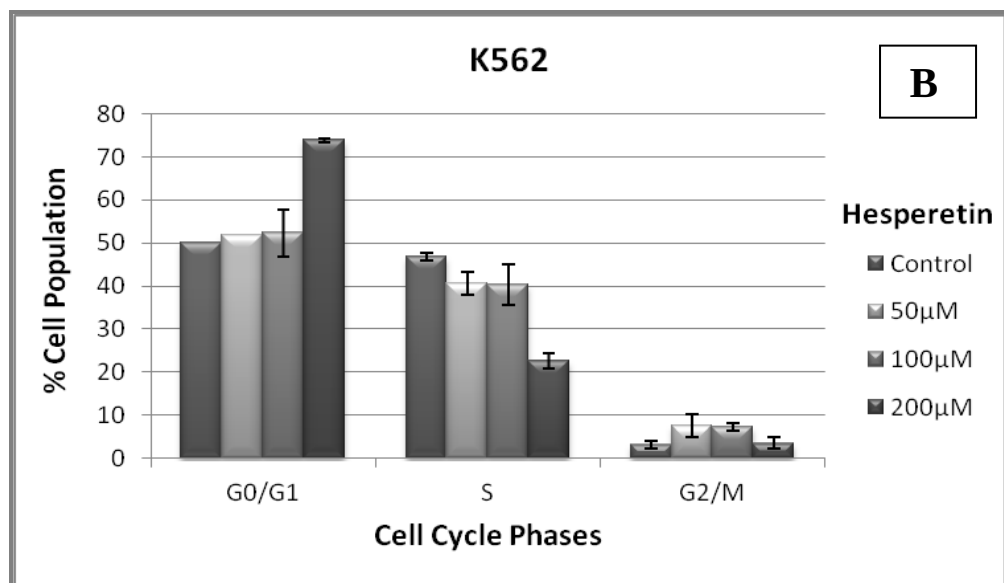


Figure 3.13 (cont.)

3.10. Identification of Genes Differentially Expressed in Fisetin and Hesperetin Treated HL60 Cells

We examined the changes in expression levels of the genes following treatment of HL60 cells with 20 and 50 μ M fisetin and 100 and 150 μ M hesperetin. Isolated total RNA was amplified and converted to biotin-labeled cRNA, which was hybridized to microarray system containing approximately 30,000 genes. Hierarchical clustering of gene expression in untreated (Control), fisetin- and hesperetin-treated HL60 cells is shown in Figure 3.14.

A total of 54 and 1608 genes were significantly regulated ($P < 0.05$) in 20 and 50 μ M fisetin treated HL60 cells, respectively. The number of genes upregulated were 30 whereas those of downregulated were 24 in 20 μ M fisetin-treated HL60 cells. In 50 μ M fisetin-treated HL60 cells, 527 genes were upregulated and 1081 genes were down regulated. Fold change analysis displayed that TXNIP (Thioredoxin-interacting protein), TFPI (Tissue factor pathway inhibitor), miRNA1974, ID1 and ID3 (Inhibitor of DNA binding 1 and 3), HSPA1B (Heat shock protein 1B) and IDH1 (Isocitrate dehydrogenase 1, NADP+) were altered genes in both 20 and 50 μ M fisetin-treated HL60 cells (Table 3.1). It was clear that MAP3K1 (Mitogen-activated protein kinase kinase kinase 1), Caspase 4 and LASS6 (Longevity assurance gene 6) were the examples of upregulated genes while LONP1 (Lon protease 1), STAT5A and STAT3 (Signal transducer and activator of transcription 5A and 3) and JAK1 (Janus kinase 1) were some of downregulated genes in 50 μ M fisetin treated HL60 cells (Table 3.1).

On the other hand, a total of 130 and 691 genes were significantly altered ($P < 0.05$) in 100 and 150 μ M hesperetin treated HL60 cells, respectively. 100 μ M hesperetin treatment resulted in the upregulation of 36 genes and downregulation of 94 genes. The number of genes upregulated were 288 whereas those of downregulated were 403 in 150 μ M hesperetin treated HL60 cells. SASH1 (SAM and SH3 domain-containing protein 1), MT1F (metallothionein 1F) and SPRR2D (small proline-rich protein 2D) were representative common upregulated genes while TUBB1 (Tubulin beta-1 chain), ID3, ID1, NMU (neuromedin U), FGFR3 (Fibroblast growth factor receptor 3) and S100P (Calcium binding protein P) were common downregulated genes in both 100 and 150 μ M hesperetin treated HL60 cells (Table 3.2) based on fold change analysis. Furthermore, 150 μ M hesperetin induced more genes that were either upregulated or downregulated as compared to 100 μ M hesperetin (Table 3.2). TXNIP, MT1A

(metallothionein 1A), MAP3K1 and SPRR2F (small proline-rich protein 2F) were some of upregulated genes while RPS25 (ribosomal protein S25) and C-MYC were the examples of downregulated genes.

3.11. Identification of Genes Differentially Expressed in Fisetin and Hesperetin treated K562 Cells

In order to determine the changes in expression levels of the genes in K562 cells treated with 50 and 100 μ M fisetin and 100 and 150 μ M hesperetin, hierarchical clustering of gene expression in untreated (Control), fisetin- and hesperetin-treated K562 cells was performed (Figure 3.15).

A total of 553 and 1734 genes were significantly regulated ($P < 0.05$) in 50 μ M and 100 μ M fisetin treated K562 cells, respectively. The number of genes upregulated were 286 whereas those of downregulated were 267 in 50 μ M fisetin treated cells. In 100 μ M fisetin treated cells, 806 genes were upregulated and 928 genes were downregulated. Both 50 and 100 μ M fisetin treatment resulted in the upregulation of common genes such as NFKBIA (Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), PMAIP1/NOXA (Phorbol-12-myristate-13-acetate-induced protein 1), CDKN1A (Cyclin dependent kinase inhibitor 1A, p21), GADD45B (Growth arrest and DNA damage inducible 45 beta) and several MT (metallothionein) genes (Table 3.3). On the other hand, it was clearly understood from Table 3.3 that each concentration of fisetin induced the upregulation of different genes in K562 cells. For instance, 50 μ M fisetin upregulated NFKBIZ (factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta) and GADD45A (Growth arrest and DNA damage inducible 45 alpha) while 100 μ M fisetin treatment resulted in the upregulation of CDKN2D (Cyclin dependent kinase inhibitor 2D, p19), TXNIP and SMAD5. Furthermore, MYC (v-myc avian myelocytomatosis viral oncogene homolog), MYB (v-myb avian myeloblastosis viral oncogene homolog), C-KIT (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog), several tubulin family members such as TUBA1A and TUBAL3 were the examples of common downregulated genes after 50 and 100 μ M fisetin treatment (Table 3.4). 100 μ M fisetin caused downregulation of several important genes such as FOXK1 (Forkhead box K1), FOXA2 (Forkhead box A2), BCL-XL (B-cell lymphoma-

extra large) and members of ABC (ATP-binding cassette) family transporters as well (Table 3.4).

On the other hand, a total of 1659 and 1201 genes were significantly changed ($P < 0.05$) in 100 and 150 μM hesperetin treated K562 cells, respectively. 100 μM hesperetin treatment resulted in the upregulation of 776 genes and downregulation of 883 genes. The number of genes upregulated were 528 whereas those of downregulated were 673 in 150 μM hesperetin treated K562 cells. As indicated in Table 3.5, DUSP1 (Dual-specificity phosphatase 1), CDKN1A, GADD45B and BCL2L11 (Bcl-2 interacting mediator, (BIM)) were the examples of common upregulated genes in 100 and 150 μM hesperetin treatment. On the other hand, 100 μM hesperetin also upregulated some other genes such as CDKN1B/ p27^{Kip1}, CASP4, DUSP5 (Dual-specificity phosphatase 5) whereas 150 μM hesperetin upregulated MT1A, DUSP3 and NFKBIA (Table 3.5). STAT5A, TUBA1A, MYB, KIT, EPCAM (Epithelial cell adhesion molecule) and PCNA (proliferating cell nuclear antigen) were the examples of common downregulated genes in 100 and 150 μM hesperetin treatment (Table 3.6). 100 μM hesperetin treatment also resulted in the downregulation of EEF1G (eukaryotic translation elongation factor 1 gamma), POLR2B (polymerase (RNA) II (DNA directed) polypeptide B) and STAT3. Furthermore, 150 μM hesperetin treatment downregulated genes such as MCM10 (minichromosome maintenance complex component 10), ABCC4 (ATP-binding cassette sub-family C member 4) and POLE2 (polymerase (DNA directed), epsilon 2) (Table 3.6).

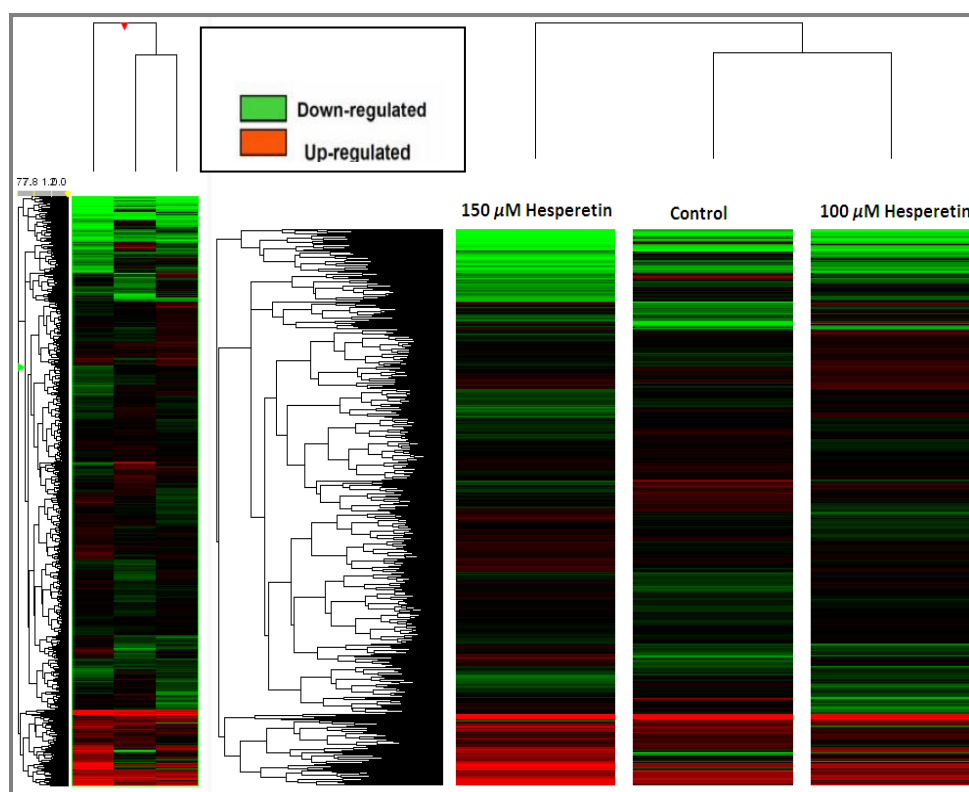
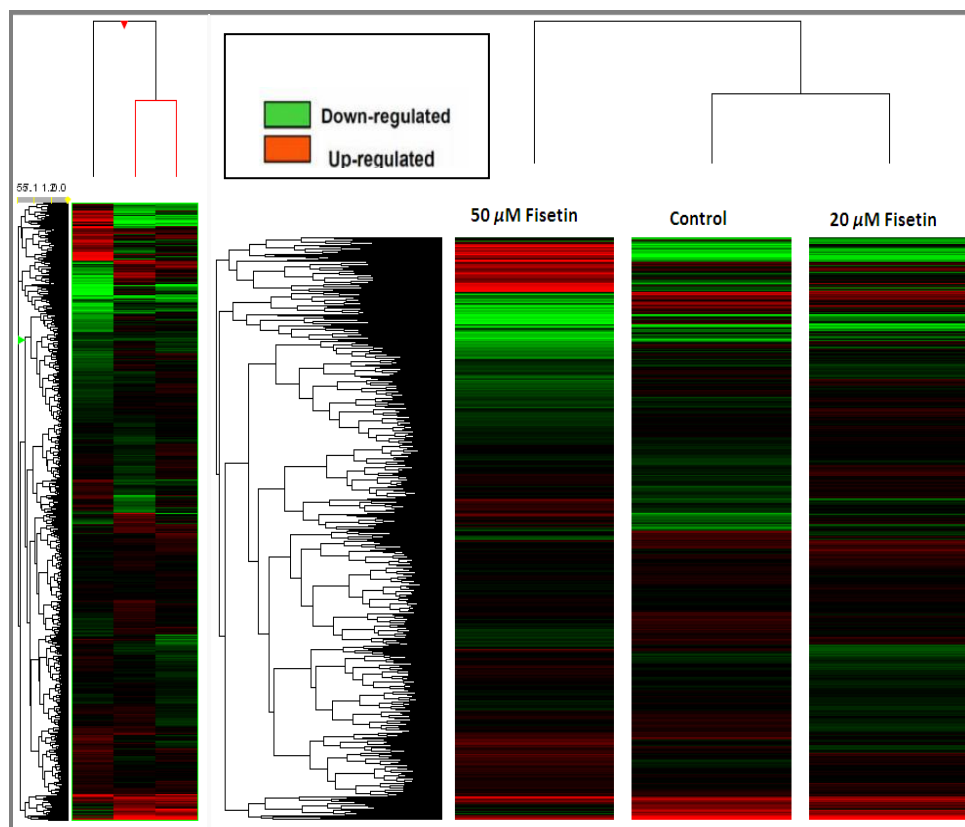


Figure 3.14. Hierarchical clustering based on gene expression data from HL60 cells exposed to fisetin (upper panel) and hesperetin (lower panel)

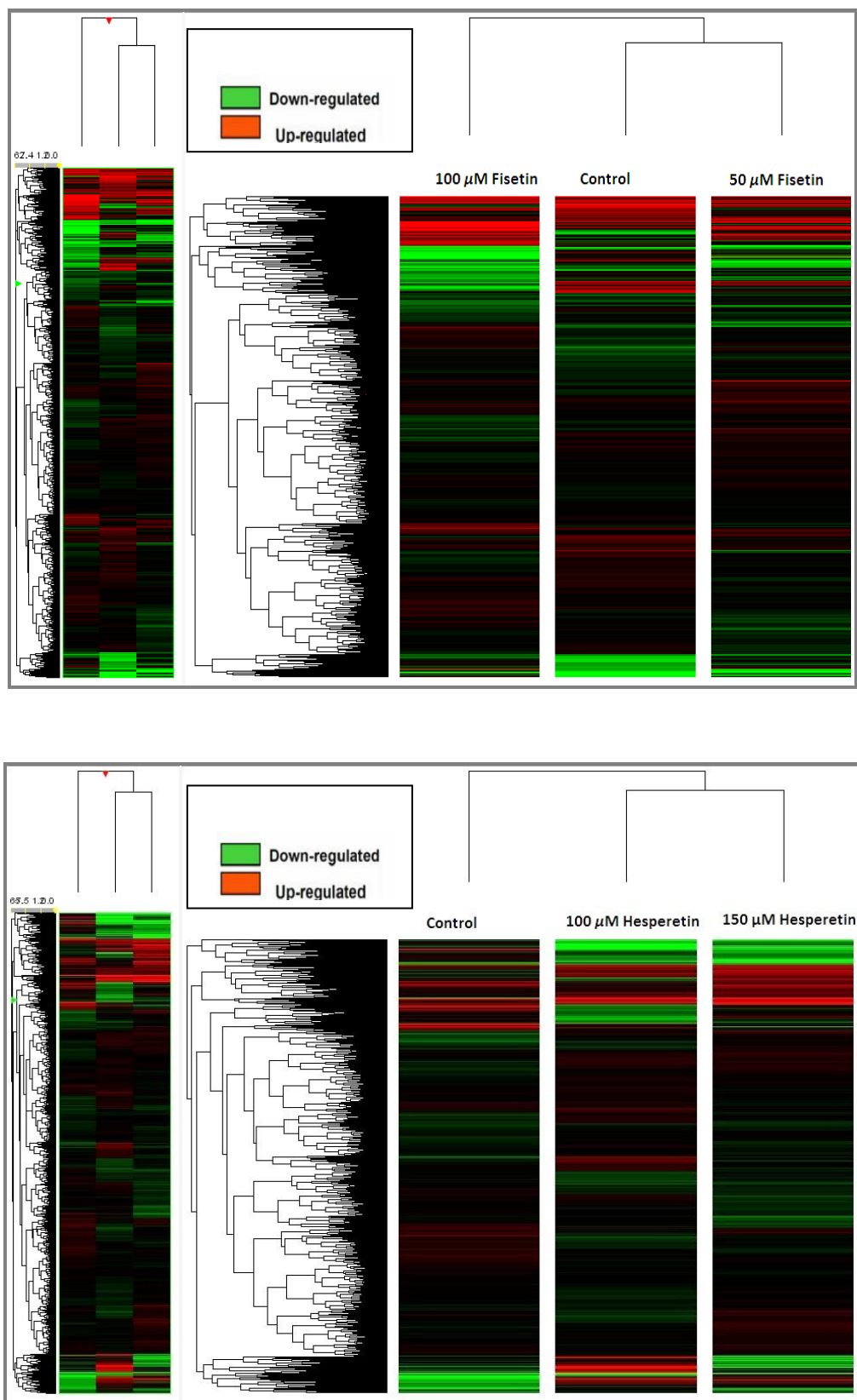


Figure 3.15. Hierarchical clustering based on gene expression data from K562 cells exposed to fisetin (upper panel) and hesperetin (lower panel)

Table 3.1. The list of genes altered ≥ 2 fold after fisetin treatment of HL60 cells (Boldface type represents upregulated genes)

Altered genes in fisetin treated HL60 cells	Fold Change		Function
	20 μ M	50 μ M	
TXNIP	11.76	12.61	Apoptosis, tumor suppressor
miRNA 1974	2.496	4.23	Inhibits cell growth
TFPI	2.055	3.680	Inhibitor of invasion and metastasis
ID3	-7.798	-8.667	Dominant negative regulators of basic helix-loop-helix transcription regulators
ID1	-3.685	-7.005	Dominant negative regulators of basic helix-loop-helix transcription regulators
HSPA1B	-3.313	-3.305	Heat shock response
IDH1	-2.685	-5.940	Cytosolic enzyme
Altered genes 50 μM fisetin treated HL60 cells	Fold Change		Function
MAP3K1	3.310		Apoptosis, caspase-3 substrate
CBLB	3.121		E3 ubiquitin ligase, autophagy
LASS6	2.728		Ceramide synthase, apoptosis
Caspase 4	2.422		Apoptosis
LONP1	-6.017		Tumor bioenergetic
STAT5A	-3.604		Cell proliferation
PHB2	-3.582		Cell survival through Ras pathway
XRCC6	-3.325		DNA repair
PIM2	-2.960		Kinase in cell survival
PRKCB	-2.920		Protumorigenic
MKNK2	-2.883		MAP kinase pathway
MAP2K5	-2.477		MAP kinase pathway
PIK3CB	-2.445		PI3 kinase pathway, cell proliferation
CHECK2	-2.382		Cell cycle
STAT3	-2.390		Cell proliferation
GAB2	-2.168		PI3 kinase pathway, cell proliferation
JAK1	-2.045		Cell proliferation

Table 3.2. The list of genes altered ≥ 2 fold after hesperetin treatment of HL60 cells (Boldface type represents upregulated genes)

Altered genes in hesperetin treated HL60 cells	Fold Change		Function
	100 μ M	150 μ M	
SPRR2D	4.574	5.985	Differentiation
MT1F	2.758	4.851	Tumor suppressor
SASH1	2.508	3.023	Tumor suppressor
S100P	-8.285	-10.153	Cell proliferation
ID1	-5.467	-6.361	Dominant negative regulators of basic helix-loop-helix transcription regulators
NMU	-5.062	-6.065	Invasion, metastasis
ID3	-3.913	-4.256	Dominant negative regulators of basic helix-loop-helix transcription regulators
FGFR3	-3.270	-3.623	Cell proliferation
TUBB1	-3.010	-3.327	Cell division, tubulin isotype
Altered genes 150 μM hesperetin treated HL60 cells	Fold Change		Function
TXNIP	2.977		Apoptosis, tumor suppressor
MT1G	2.939		Tumor suppressor
TPM1	2.734		Tropomyosin 1, tumor suppressor
SPRR2F	2.673		Differentiation
RERG	2.629		Ras related growth inhibitor
ST7	2.609		Tumor suppressor, cell cycle arrest
MAP3K1	2.606		Apoptosis, caspase-3 substrate
RASA1	2.438		Inhibitor of mitogenic signal
MT1A	2.434		Tumor suppressor
SIN3A	2.400		Tumor suppressor
RPS25	-8.177		Ribosome component
TUBA1C	-3.544		Cell division, tubulin isotype
EEF1A1	-3.042		Protein translation
RPS6P1	-3.029		Ribosome component
PRKCB1	-2.432		Cell proliferation
C-MYC	-2.157		Proto-oncogene Transcription factor
PIM1	-2.330		Proto-oncogene kinase
TUBB2C	-2.226		Cell division
RPS9	-2.208		Ribosome component
PCK2	-2.206		Enzyme involved in gluconeogenesis
RXRA	-2.122		Differentiation

Table 3.3. The list of genes upregulated ≥ 2 fold after fisetin treatment of K562 cells

Upregulated genes in fisetin treated K562 cells	Fold Change		Function
	50 μ M	100 μ M	
NFKBIA	4.622	2.569	NFKB inhibitor, cell growth inhibition
MT1A	3.553	19.505	Tumor suppressor
NOXA	2.614	2.457	Apoptosis
CDKN1A/P21/WAF1/CIP1	2.217	2.298	Cell cycle, growth arrest
TP53TG3	2.194	3.789	Growth inhibition
MT1G	2.178	39.13	Tumor suppressor
GADD45B	2.108	2.888	Cell cycle, growth arrest, apoptosis
MT2A	2.057	8.358	Tumor suppressor
Upregulated genes 50 μM fisetin treated K562 cells	Fold Change		Function
FAT1	2.862		Tumor suppressor
TNFAIP3	2.793		Tumor suppressor
IRF1	2.691		Transcription factor
NFKBIZ	2.678		NFKB inhibitor, cell growth inhibition
BTG1	2.501		Antiproliferative, differentiation
GADD45A	2.206		Cell cycle, growth arrest, apoptosis
Upregulated genes in 100 μM fisetin treated K562 cells	Fold Change		Function
MT1E	8.703		Tumor suppressor
MT1F	8.336		Tumor suppressor
miR1974	7.484		Inhibits cell growth
OKL38	6.442		Inhibits cell growth
MT1X	5.931		Tumor suppressor
MT1H	4.320		Tumor suppressor
TXNIP	3.428		Apoptosis, tumor suppressor
p19/CDKN2D	3.169		Cell cycle, growth arrest
PTPLB	3.05		Protein phosphatase
RASSF1	2.609		Tumor suppressor
MED23	2.498		Apoptosis
SMAD5	2.167		TGF- β -induced anti-proliferative effect

Table 3.4. The list of genes downregulated ≥ 2 fold after fisetin treatment of K562 cells

Downregulated genes in fisetin treated K562 cells	Fold Change		Function
	50 μ M	100 μ M	
S100P	-4.223	-4.460	Cell proliferation
ID3	-4.006	-3.063	Dominant negative regulators of basic helix-loop-helix transcription regulators
TUBA1A	-3.470	-5.619	Cell division
NMU	-3.365	-7.353	Invasion, metastasis
KIF1A	-3.112	-4.321	Organelle movement
TUBA1C	-2.881	-4.169	Cell division
ID1	-2.808	-3.00	Dominant negative regulators of basic helix-loop-helix transcription regulators
EPCAM	-2.805	-2.578	Adhesion
ICAM2	-2.794	-2.928	Adhesion
EGR1	-2.693	-4.77	Cell growth
STAT5A	-2.633	-4.804	Cell proliferation
SERPINH1	-2.383	-3.190	Invasion, metastasis
C-KIT	-2.276	-9.460	Oncogene
FGFR3	-2.142	-2.151	Cell proliferation
MYB	-2.137	-9.736	Oncogene
SERPINB1	-2.131	-3.287	Invasion, metastasis
STAT3	-2.107	-2.129	Cell proliferation
TUBAL3	-2.106	-3.323	Cell division
MYC	-2.048	-6.068	Proto-oncogene, Transcription factor
PRKCB1	-2.043	-5.272	Cell proliferation
VEGFB	-2.018	-2.289	Tumour angiogenesis
TUBB6	-2.011	-2.644	Cell division
Downregulated genes in 100 μ M fisetin treated K562 cells	Fold Change		Function
PDCD61P	-8.743		Apoptosis blocker
FO XK1	-4.357		Cell proliferation
ABCC4	-4.255		Transporter
BCL-XL	-4.188		Antiapoptotik
PIM1	-4.125		Oncogene kinase
MAPK1	-3.30		MAPK pathway
IDH1	-3.251		Cytosolic enzyme
TRAP1	-3.142		Mitochondrial chaperone, antiapoptotic
FOXA2	-2.983		Cell proliferation
ABCB7	-2.772		Transporter
SERPINB6	-2.715		Invasion, metastasis
TUBB2C	-2.620		Cell division
STAT5B	-2.600		Cell proliferation
LAMA5	-2.474		Adhesion
PIM2	-2.338		Oncogene kinase
EIF4G2	-2.168		Translation
PRKCA	-2.146		Cell growth, invasion

Table 3.5. The list of genes upregulated ≥ 2 fold after hesperetin treatment of K562 cells

Upregulated genes in hesperetin treated K562 cells	Fold Change		Function
	100 μ M	150 μ M	
DUSP1	10.703	5.483	Protein phosphatase
CDKN1A/P21/WAF1/CIP1	5.605	3.978	Cell cycle, growth arrest
GADD45B	5.358	2.255	Cell cycle, growth arrest, apoptosis
SPRR2D	5.310	6.030	Differentiation
BCL2L11	5.265	2.056	Apoptosis facilitator
OKL38	4.944	5.795	Inhibits cell growth
MT1F	3.834	8.3	Tumor suppressor
DUSP10	3.74	2.354	Protein phosphatase
PPP1R15A	3.223	2.354	Growth arrest
BTG1	3.044	2.417	Antiproliferative, differentiation
GADD45G	3.018	4.517	Cell cycle, growth arrest, apoptosis
CDKN2D/p19	2.949	3.442	Cell cycle, growth arrest
TXNIP	2.642	2.449	Apoptosis, tumor suppressor
PIK3IP1	2.614	2.509	Negative regulator of PI3K
Upregulated genes 100 μM hesperetin treated K562 cells	Fold Change		Function
FILIP1L	7.429		Cell migration inhibitor
miRNA 1974			Inhibits cell growth
CDKN1B/ p27 ^{Kip1}	3.955		Cell cycle, growth arrest
CBLB	3.523		E3 ubiquitin ligase, autophagy
DUSP5	3.333		Protein phosphatase
E2F6	3.042		Antiproliferative transcription factor
SIN3A	2.825		Tumor suppressor
CASP4	2.402		Apoptosis
Upregulated genes in 150 μM hesperetin treated K562 cells	Fold Change		Function
MT1A	2.77		Tumor suppressor
NFKBIA	2.624		Cell growth inhibition
CDKN2AIP	2.509		Cell cycle, growth arrest
DUSP3	2.040		Protein phosphatase

Table 3.6. The list of genes downregulated ≥ 2 fold after hesperetin treatment of K562 cells

Downregulated genes in hesperetin treated K562 cells	Fold Change		Function
	100 μ M	150 μ M	
STAT5A	-5.904	-2.530	Proliferation
TUBA1A	-5.312	-2.545	Cell division
MYB	-4.605	-15.53	Oncogene
FGFR3	-4.308	-4.618	Cell proliferation
ICAM2	-3.779	-3.509	Adhesion
KIF1A	-3.708	-4.125	Organelle movement
EGR1	-3.517	-5.063	Proliferation
KIT	-3.494	-5.595	Oncogene
MCM3	-3.438	-2.401	Replication initiation
E2F2	-3.425	-5.054	Cell cycle
MYC	-3.394	-3.131	Proto-oncogene Transcription factor
NMU	-3.222	-5.343	Invasion, metastasis
MAT2A	-3.135	-5.138	Cell growth
POLD1	-3.083	-2.215	Replication, repair
TRAP1	-3.078	-2.031	Proliferation, antiapoptotic
TUBAL3	-2.893	-3.301	Cell division
SERBP1	-2.803	-3.622	Invasion, metastasis
PRKCB	-2.542	-3.312	Protumorigenic
EPCAM	-2.427	-2.00	Adhesion
E2F3	-2.399	-2.217	Cell growth and differentiation
EIF2S2	-2.362	-2.537	Translation initiation
S100P	-2.302	-3.890	Cell proliferation
PCNA	-2.194	-2.735	Replication
KIF11	-2.011	-2.609	Organelle movement, Mitosis
Downregulated genes in 100 μ M hesperetin treated K562 cells	Fold Change		Function
EEF1G	-7.328		Translation elongation
EIF4G1	-4.362		Translation initiation
POLR2B	-2.350		Transcription
STAT3	-2.217		Proliferation
LONP1	-2.186		Proliferation, metastasis
POLR2A	-2.115		Transcription
Downregulated genes in 150 μ M hesperetin treated K562 cells	Fold Change		Function
MCM10	-3.640		Replication initiation
ABCC4	-2.782		Transporter
MCM7	-2.666		Replication initiation
POLE2	-2.661		Replication
EIF5	-2.640		Translation initiation
MCM4	-2.436		Replication initiation
MCM2	-2.367		Replication initiation

3.12. Identification of Genetic Networks Affected by Fisetin and Hesperetin in HL60 Cells

To examine the affected genetic networks after fisetin and hesperetin treatment, we carried out pathway analysis using the IPA tool. These networks describe functional relationships between gene products based on known interactions in the literature. The results showed that MAPK signaling pathway, ID signaling pathway, cell cycle pathway, JAK/STAT signaling pathway, cell cycle check point pathways and PI3K/AKT signaling pathways were examples of the most altered networks in fisetin treated HL60 cells (Table 3.7).

Table 3.7. Affected networks in fisetin-treated HL60 cells. (Boldface type represents networks modulated in both 20- and 50- μ M fisetin treatment)

NETWORK/PATHWAY	P VALUE
MAPK signaling pathway (50 μ M)	0.04
ID signaling pathway	2.71E-05 (20 μ M) 3.62E-04 (50 μ M)
Nucleotide excision repair (50 μ M)	1.48E-06
Cell cycle pathway (50 μ M)	2.47E-05
JAK/STAT signaling pathway (50 μ M)	0.002
Protein Folding	0.01 (20 μ M) 2.01E-09 (50 μ M)
Cell cycle check point pathways (50 μ M)	2.57E-04
PI3K/AKT pathway (50 μ M)	0.04

Based on the data obtained from the IPA analysis, ID signaling pathway, eukaryotic ribosome and translation related networks, gluconeogenesis related and mitosis related networks, TGF- β pathway and MAPK pathway were the affected signaling networks in hesperetin-treated HL60 cells (Table 3.8).

Table 3.8. Affected networks in hesperetin-treated HL60 cells. (Boldface type represents networks modulated in both 100- and 150- μ M hesperetin treatment)

NETWORK/PATHWAY	P VALUE
ID signaling pathway	4.52E-04 (100 μ M) 0.008 (150 μ M)
TGF-β pathway	7.45E-04 (100 μ M) 3.32E-04 (150 μ M)
Cytoplasmic ribosomal components (150 μ M)	1.27E-10
Translation factors (150 μ M)	4.24E-06
Gluconeogenesis (150 μ M)	4.29E-04
Mitosis (150 μ M)	0.001
MAPK signaling pathway (150 μ M)	0.01

3.13. Identification of Genetic Networks Affected by Fisetin and Hesperetin in K562 Cells

IPA analysis was performed to identify modulated networks based on the functional relationships between gene products by fisetin and hesperetin in K562 cells (Table 3.9). Apoptosis modulation and signaling, TP53 network, TNF- α signaling, KIT receptor signaling pathway, JAK/STAT signaling, adhesion networks, growth hormone receptor signaling and angiogenesis were modulated networks in fisetin-treated K562 cells.

Hesperetin treatment in K562 cells resulted in alterations in translation initiation and elongation networks, replication networks, transcription networks, EGF (epidermal growth factor) signaling, JAK/STAT signaling, KIT receptor signaling pathway, growth hormone receptor signaling and PI3K pathway (Table 3.10).

Table 3.9. Affected networks in fisetin-treated K562 cells. (Boldface type represents networks modulated in both 50- and 100- μ M fisetin treatment)

NETWORK/PATHWAY	P VALUE
Apoptosis modulation and signaling	9.46E-06 (50 μ M) 0.04 (100 μ M)
TP53 network	0.003 (50 μ M) 0.02 (100 μ M)
KIT receptor signaling	1.06E-06 (50 μ M) 1.86E-08 (100 μ M)
JAK/STAT signaling	0.001 (50 μ M) 1.63E-05 (100 μ M)
Adhesion networks	0.004 (50 μ M) 4,09E-06 (100 μ M)
Growth hormone receptor signaling	0.004 (50 μ M) 2,00E-05 (100 μ M)
Angiogenesis (50 μ M)	0.02
TNF- α signaling (50 μ M)	1,44E-05

Table 3.10. Affected networks in hesperetin-treated K562 cells. (Boldface type represents networks modulated in both 100- and 150- μ M hesperetin treatment)

NETWORK/PATHWAY	P VALUE
Translation initiation	5,39E-12 (100 μ M) 0.001 (150 μ M)
Translation elongation	0.002 (100 μ M) 0.001 (150 μ M)
Replication	1,18E-05 (100 μ M) 8,01E-09 (150 μ M)
Apoptosis modulation	0.002 (100 μ M) 0.01 (150 μ M)
JAK/STAT signaling	0.001 (100 μ M) 0.02 (150 μ M)
KIT receptor signaling	1,37E-05 (100 μ M) 2,06E-05 (150 μ M)
Growth hormone receptor signaling	0.04 (100 μ M) 0.02 (150 μ M)
PI3K pathway	0.04 (100 μ M) 0.03 (150 μ M)

CHAPTER 4

CONCLUSION

Chronic myeloid leukemia (CML) is defined by a reciprocal translocation between the breakpoint cluster (BCR) gene on chromosome 22 and the abelson leukemia virus oncogene (ABL) gene on chromosome 9, which encodes a fusion oncoprotein BCR-ABL1 with constitutive tyrosine kinase activity (Quinta's-Cardama and Cortes 2009). At present, the development and usage of TKIs provide improved outcomes in CML patients.

Acute promyelocytic leukaemia (APL), a subtype of AML, is characterized by specific chromosomal translocations between the retinoic acid (RA) receptor α (RAR α) gene on chromosome 17 and any gene located on different chromosomes in order to make a variety of X-RAR α fusions. In most cases, translocation, t(15;17), between the retinoic acid receptor-alpha (RAR α) gene on chromosome 17 and the promyelocytic leukaemia (PML) gene on chromosome 15 is very common. Chemotherapy, ATRA, ATO, monoclonal antibodies and haematopoietic stem cell transplantation are the options for the treatment of AML patients (de Thé, et al. 2012).

The heterogenic nature and differences in biological characteristics of leukemias and development of resistance in most cases make them difficult to treat. They remain incurable although several treatment options exist for CML and APL as explained. Thus, novel treatment approaches are required to improve outcome.

Flavonoids are a significant member of polyphenolic compounds that are commonly found in the human diet. They are widely distributed in the plant species such as fruits and vegetables. Currently, there is an increased interest in polyphenolics, including flavonoids, because data obtained from clinical and experimental studies display that they possess many therapeutic features including antioxidant, antiinflammatory, cardioprotective, and anticarcinogenic activities. Flavonoids are known to prevent the stages of cancer development by causing alterations in various signaling pathways acting in cell proliferation, differentiation, apoptosis, angiogenesis and metastasis. Therefore, both natural and synthetic analogs are being investigated for their potentials in cancer therapy (Ravishankar, et al. 2013).

Fisetin, hesperetin and vitexin, naturally occurring plant flavonoids, have been found to induce apoptosis in various tumor cells such as colon cancer, breast and pancreatic cancer. However, there are no studies on K562 CML cells and HL60 APL cells and the mechanisms regulating fisetin, hesperetin and vitexin-induced effects in these leukemia cells remain elusive.

This study was conducted to examine the cytotoxic, cytostatic, and apoptotic effects of fisetin, hesperetin and vitexin on K562 CML and HL60 APL cells. The cells were treated with increasing concentrations of these three agents (1-200 μ M) and their cytotoxic/antiproliferative effects were determined by MTT cell proliferation assay. The results of this assay revealed that fisetin, hesperetin and vitexin decreased proliferation of the both CML and APL cells in a dose- and time-dependent manner (Figure 3.1 - 3.3). On the other hand, Annexin-V/PI double staining showed increases in apoptotic cell population in both K562 and HL60 cells in response to increasing concentrations of agents as compared to untreated controls (Figure 3.4 - 3.7). Based on the data obtained by flow cytometry, it could be concluded that fisetin triggers the strongest apoptosis in a dose- dependent manner among other flavonoids in both K562 and HL60 cells while vitexin is the less effective agent. To elucidate the molecular mechanism responsible for apoptosis in K562 and HL60 cells, we first checked the effects of fisetin, hesperetin and vitexin on MMP (Figure 3.8 - 3.9). The JC-1 dye-based assay results revealed that all agents caused loss of MMP in a dose-dependent manner in both cells. It is very well known that mitochondria functions for all apoptotic pathways and it is widely accepted that changes in the structure and function of mitochondria play an important role in caspase-dependent apoptosis. Caspase-3 functions in the last step of caspase-mediated apoptosis and is responsible for the properties of apoptotic cells (such as DNA fragmentation) (Wang, et al. 1999). Based on this knowledge, we next checked the activation of caspase-3 enzyme in treated K562 and HL60 cells. Our data demonstrated that fisetin and hesperetin- treated HL60 cells displayed dose-dependent increases in caspase-3 enzyme activity whereas there were very small changes in caspase-3 enzyme activity of HL60 cells treated with vitexin (Figure 3.10). Caspase-3 enzyme activity increased in fisetin-treated K562 cells in a dose-dependent manner. However, hesperetin and vitexin treatment caused changes in caspase-3 activity at only the highest concentration (2.8-fold and 1.4-fold at 200 μ M, respectively) (Figure 3.11). Taken together, we can conclude that fisetin may induce apoptosis in both cells through the loss of MMP and caspase-3 activation at all concentrations while hesperetin and vitexin

treatments were most effective at the highest concentration to induce apoptosis through mitochondrial pathway.

Similar to our results, fisetin was found to induce depolarization in mitochondrial membrane and caspase activation in HCT-116 colon cancer (Lim do and Park 2009) and DU145, PC-3 and LNCaP prostate cancer cells (Szliszka, et al. 2011). In U266 multiple myeloma cells and U967 leukemic cells underwent apoptosis in response to fisetin through caspase-3 activation (Jank, et al. 2012, Monasterio, et al. 2004). In a recent study, fisetin induced apoptosis in the human nonsmall cell lung cancer cell line, NCI-H460, via mitochondrial membrane depolarization and caspase-3 activation (Kang, et al. 2014).

Hesperetin was displayed to attenuate mitochondrial membrane potential with increased expression of caspase-3, causing apoptotic in SiHa cells cervical cancer cells (Alshatwi, et al. 2013). In another study, hesperetin also induced apoptosis on HT-29 colon cancer cells by causing increases in cleaved caspase-3 expression (Sivagami, et al. 2012). Only one study including HL60 cells displayed that hesperetin showed cytotoxic activity and could activate caspase-3 in a dose-dependent manner (Chen, et al. 2003). In this study, HL60 cells treated with 20-, 40- and 80- μ M hesperetin and caspase-3 activity was detected using Ac-DEVD-pNA as a substrate like our data.

The effects of vitexin on human U937 leukemia cells were found to be related with the induction of apoptosis via mitochondrial pathway, since caspase-3 activity increased. It was found that vitexin reduced the proportion of viable U937 human leukemia cells in a concentration- and time-dependent manner. The concentration required to inhibit cell growth by 50% was about 200.34 μ M (Lee, et al. 2012).

We also investigated the cytostatic property of fisetin, hesperetin and vitexin on CML and APL cells by flow cytometry. The treatment of HL60 cells with fisetin and hesperetin resulted in G2/M arrest in a dose-dependent manner. Hesperetin also inhibited the cell cycle progression in G0/G1 phase. On the other hand, vitexin treatment was responsible for cell cycle arrest in both G2/M and G0/G1 phases only at the highest concentration compared to untreated controls (Figure 3.12). The cell cycle progression of fisetin-treated K562 cells was found to be arrested in both S and G2/M phases while hesperetin blocked the cell cycle progression in G0/G1 phase with significant accumulation of the cells at the highest concentration. Furthermore, the percentage of the cells in the G0/G1 and G2/M phases increased after vitexin treatment (Figure 3.13). According to the results obtained, it could be concluded that fisetin,

hesperetin and vitexin affected different phases of the cell cycle based on the cell type and affected cell cycle regulators such as cyclins and cyclin-dependent kinases, therefore inhibited cell growth.

In the literature, there are various studies showing the effects of these three flavonoids on the cell cycle in different cancer types. Similar to the results (fisetin arrested cell cycle progression in G2/M in both CML and APL cells) presented in this study, fisetin induced G2/M arrest in human epidermoid carcinoma A431 cells (Pal, et al. 2013). Fisetin also caused the inhibition of cell cycle progression in G2/M in HT-29 colon cancer cells by decreasing the activities of cell cycle regulator proteins including cyclin-dependent kinases such as cdk-2 and -4 and several cyclins (Lu, et al. 2005). The treatment of human prostate cancer PC3 cells resulted in G2/M arrest (Haddad, et al. 2006). The G2/M checkpoint is important to prevent cells with damaged DNA from entering mitosis. Therefore, this checkpoint gives enough time to those cells in order to repair DNA and stop their proliferation. Thus, the G₂/M phase arrest is one of the most critical checkpoints of many anticancer agents in cancer cells (Lu, et al. 2005, Haddad, et al. 2006). As well as G2/M arrest, fisetin also caused G0/G1 arrest in T24 and E7 bladder cancer cells by increasing the expression of p53 and p21 proteins, and decreasing the levels of cyclin D1, cyclin A, Cdk-4 and Cdk-2, thereby contributing to cell cycle arrest (Li, et al. 2011). We also determined that fisetin treatment caused S phase arrest in K562 CML cells for the first time.

Hesperetin treatment resulted in G0/G1 arrest in both CML and APL cells in our study. Similarly, hesperetin treatment resulted in significant cell cycle arrest in the G1 phase by downregulating Cdk-2 and -4 together with cyclin D in MCF-7 breast cancer cells (Choi 2007). It is known that G1 arrest results in repair of DNA or apoptosis (Choi 2007). Therefore, this arrest could inhibit cell proliferation and contribute the apoptotic process as a possible mechanism of hesperetin action. In hesperetin treated HL60 APL cells, G2/M arrest was also found to be effective in our study. Human cervical SiHa cells treated with hesperetin displayed G2/M arrest in a dose-dependent manner as well (Alshatwi, et al. 2013).

Vitexin was displayed to block cell cycle progression in both G0/G1 and G2/M phases for both CML and APL cells. Similarly, vitexin inhibited growth and induced cell cycle arrest at G1/G0 in HepG2 hepatocellular carcinoma cells (Wang, et al. 2014). However, there is no study showing the effect of vitexin on G2/M arrest.

In this study, we also performed genome-wide microarray analysis in K562 and HL60 cells to identify the genes and networks that are involved in fisetin- and hesperetin-related antiproliferative, apoptotic and cytostatic effects. For this purpose, heat map analysis was performed to cluster the genes in K562 and HL60 cells exposed to fisetin and hesperetin. Moreover, the list of differentially expressed genes constrained by P-value <0.05 and at least 2.0 fold change was indicated. In order to demonstrate gene profiling in response to fisetin and hesperetin, the Illumina Human HT-12v4 microarray assay was used.

A total of 54 and 1608 genes were detected to be significantly either up- or down-regulated ($P < 0.05$) by 20 and 50 μM fisetin treatment in HL60 cells, as compared to untreated controls, respectively. As summarized in Table 3.1, 20 and 50 μM fisetin treated- HL60 cells shared common genes, which were more than 2.0 fold up- or down-regulated. For instance, TXNIP, miRNA1974 and TFPI genes were common upregulated genes and they are known to be involved in the inhibition of cell growth, invasion and metastasis. TXNIP or thioredoxin interacting protein plays a crucial role in apoptosis and oxidation. Its expression was found to be downregulated in many tumor cells (Escrich, et al. 2004) and overexpression of TXNIP inhibits cancer cell growth (Yamaguchi, et al. 2008, Yamaguchi, et al. 2014). Tissue factor pathway inhibitor (TFPI) acts as an inhibitor of plasmin activating matrix metalloproteinases (MMPs) that degrades the extracellular matrix (Lavergne, et al. 2013). Therefore, its overexpression makes TFPI a potential blocker of tumor invasion and metastasis. miRNA1974 was shown to be downregulated in adrenocortical carcinoma and its overexpression reduced cell proliferation and induced cell death (Özata, et al. 2011). In conclusion, TXNIP, TFPI and miRNA1974 were thought to cause fisetin-induced cell death. Downregulation of some common genes such as ID1, ID3, HSPA1B and IDH1 in 20 and 50 μM fisetin treated-HL60 cells could be responsible for its apoptotic effects as well. ID proteins regulate the basic helix-loop-helix transcription regulators in a negative manner and are involved in various cellular processes including differentiation, proliferation, metastasis, apoptosis, angiogenesis, and neoplastic transformation (Norton 2000). In a recent study, suppression of ID1 and ID3 was related to reduced tumor growth, which was characterized by decreases in angiogenesis and increases in apoptosis (Chen, et al. 2014). The HSPA1B codes for a 70 kDa heat shock protein, which inhibits the accumulation of protein aggregates and protects the cells against DNA damage, thus promoting cell survival under stressful conditions (Guo, et al. 2011). Therefore, its

downregulation could be a reason for fisetin-induced antiproliferative effect on HL60 cells. The IDH1 encodes NADP⁺ dependent cytosolic isocitrate dehydrogenase to produce NADPH for several cellular processes such as protecting cells from reactive oxygen species (ROS). Therefore, its downregulation after fisetin treatment might cause cell death due to the accumulation of ROS that damage proteins, lipids and DNA in the cells (Kim, et al. 2014). As well as these common altered genes, 50 μ M fisetin also induced either up- or down-regulation of several genes as indicated in Table 3.1. These genes were found to encode proteins with significant functions in important cancer related signaling pathways such as MAPK pathway, PI3K pathway and JAK/STAT pathway. For instance, STAT5A, STAT3 and JAK1, important players of JAK/STAT signaling pathway responsible for leukemic cell growth and differentiation, were found to be downregulated after fisetin treatment. It has been known that downregulation of STAT5 resulted in the downregulation of its targets such as Bcl-xL, cyclinD1, and c-myc at both mRNA and protein levels (Wang, et al. 2011), thus inducing apoptosis. MKNK2 (MAP kinase interacting serine/threonine kinase 2) and MAP2K5 (mitogen-activated protein kinase kinase 5) were other downregulated genes involved in MAPK pathway-related oncogenic transformation and malignant cell proliferation, thus their downregulation could result in apoptosis of HL60 cells. Two different genes playing significant roles in PI3K pathway, PIK3CB (catalytic subunit of phosphoinositide 3-kinase (PI3K)) and GAB2, were also downregulated after fisetin treatment, which might lead to suppression of leukemic cell growth and induction of apoptosis. MAP3K1 was upregulated in 50 μ M fisetin treated HL60 cells although it is involved in MAPK pathway triggering cell survival. However, in a recent study, MAP3K1 was found to induce apoptosis via its C-terminal kinase domain after its caspase-3 mediated cleavage (Pham, et al. 2013). LASS6 and caspase-4 were other examples of fisetin-induced upregulated genes. LASS6, member of the longevity-assurance homologue family, function in ceramide generation, which is known to induce cell death (Walker, et al. 2010). Caspase-4 (also called caspase 11) induced cell apoptosis when overexpressed (Nq and Monack 2013). In conclusion, fisetin triggers apoptosis and growth suppression via affecting various significant targets as discussed in HL60 cells and these new targets could open the usage of new strategies together with fisetin to overcome difficulties in APL treatment. These data also provide significant insights about the mechanism of action of fisetin in HL60 cells.

Moreover, HL60 cells treated with 100 and 150 μM hesperetin changed the expression of 130 and 691 genes ($P < 0.05$), respectively. As indicated in Table 3.2, 100 and 150 μM hesperetin-treated HL60 cells shared common genes, which were more than 2.0 fold up- or down-regulated. SPRR2D, MTF1 and SASH1 were upregulated genes that are known to play roles in cell proliferation, apoptosis, differentiation. SPRRs, small proline-rich proteins, are involved in cell differentiation process. Cancer cells have no or lower expression of SPRRs compared to normal cells (Anisowicz, et al. 1999). Therefore, SPRR2D and also SPRR2F (at 150 μM hesperetin) were increased after hesperetin treatment, thus somehow inducing cell growth suppression in HL60 cells. On the other hand, MTF1, a member of metallothionin family, is expressed transiently in response to stimulation and plays an important role in cell growth by modulating gene transcription in a positive or negative manner depending on the cell types (Lu, et al. 2003). In a recent study, MTF1 gene was shown to be downregulated in colon cancer cells and its exogenous overexpression increased apoptosis (Yan, et al. 2012). In our study, hesperetin induced upregulation of MTF1, therefore inducing growth suppression in HL60 cells. SASH1 is called as a potential tumor suppressor in several cancer types and may function in various signaling pathways due to its SH3 and SAM domains. Its downregulation is related to tumor growth (Meng, et al. 2013). Therefore, its increased expression after hesperetin treatment might induce apoptosis and growth suppression. Similar to fisetin treatment as discussed above, ID1 and ID3 genes were downregulated in 100 and 150 μM hesperetin treatment, thus inducing apoptosis. The other common downregulated gene was NMU, which was found to be increased in AML cells. Its knockdown caused growth arrest and it may be a reason for hesperetin-related antiproliferative effect (Shetzline, et al. 2004). TUBB1 encodes a member of beta tubulin protein family and it is known that alpha and beta tubulins heterodimerize to form microtubules that play important roles in mitosis (Leandro-García, et al. 2010). Therefore, hesperetin treatment downregulated TUBB1 gene and could arrest cells in mitosis and finally cause cell death. As well as these common altered genes, 150 μM hesperetin also induced either up- or down-regulation of several genes as indicated in Table 3.2. It is interpreted from Table 3.2 that increased hesperetin concentration resulted in downregulation of additional tubulin encoding genes, TUBB2C and TUBA1A, which could cause problems in cell division and then induce apoptosis. 150 μM hesperetin impaired protein synthesis by targeting cytoplasmic ribosomal proteins such as RPS6P1, RPS9 and RPS25 and factors involved in translation initiation like

EEF1A1. Another important downregulated gene was C-MYC which is described as a proto-oncogenic transcription factor and upregulated in various cancer types such as leukemia, lung and breast cancers (Dang 1999). C-MYC is responsible for cell proliferation and inhibition of apoptosis via altering the expression of several target genes (Akinyeke, et al. 1999). Therefore, its downregulation by hesperetin could be a strategy to inhibit HL60 cell growth. PIM1, a member of serine/threonine kinases involved in cell survival and proliferation, is overexpressed in a variety of cancer types such as leukemias, lymphomas, pancreatic and prostate cancers (Saurabh, et al. 2014). It is also known that PIM1 functions together with C-MYC to induce malignancy. In our study, hesperetin downregulates both C-MYC and PIM1, therefore inducing significant growth inhibition and apoptosis.

A total of 553 and 1734 genes were significantly regulated ($P < 0.05$) in 50 μM and 100 μM fisetin treated K562 cells, respectively. The upregulated genes after fisetin treatment were indicated in Table 3.3. As clearly seen, there were common upregulated genes after 50 μM and 100 μM fisetin treatment. NFKBIA, PMAIP1/NOXA, CDKN1A and GADD45B were examples of the genes upregulated in both 50 μM and 100 μM fisetin treated K562 cells with significant effects on cell growth and cell death. For instance, NFKBIA (also known as $\text{I}\kappa\text{B}\alpha$), a member of the NF-kappa-B inhibitor family, inhibits NF- κB pathway which is responsible for the transcription of several genes involved in cell survival (Li and Sethi 2010). Therefore, upregulation of this gene by fisetin could suppress cell growth and induce apoptosis. The BH3-only protein PMAIP1 (NOXA) is a p53 transcriptional target in response to DNA damage and has been reported to be involved in chemotherapeutic agent-induced apoptosis (Zhao, et al. 2014). Its upregulation may result in apoptosis of K562 cells after fisetin treatment. The loss of cell cycle control is associated with malignant transformation. CDKN1A ($\text{p}21^{\text{WAF1/Cip1}}$), one of the regulators involved in cell cycle control, is responsible for G1 arrest in response to stimuli and inhibits tumor growth. p21 acts by binding to and inhibiting the cyclin-dependent kinases (CDKs) CDK2 and CDK1 (Abbas and Dutta 2009). Similarly, 100 μM fisetin upregulated CDKN2D (p19) that induces cell cycle arrest by inhibiting CDK4 and CDK6. Fisetin treatment upregulated CDKN1A and could induce growth arrest. Fisetin induced upregulation of GADD45B and GADD45A (for 50 μM fisetin treatment) participating in cell cycle arrest, DNA repair, cell survival and apoptosis could be another important event to suppress leukemic cell growth (Liebermann, et al. 2011). 100 μM fisetin treatment resulted in upregulation of

specific genes such as MED23 and SMAD5. MED23 (mediator complex subunit 23) is involved in RNA polymerase II-dependent transcription initiation together with basic transcription factors. Recently, it is found to be related to several cellular processes such as cell survival, proliferation and carcinogenesis. MED23 is downregulated in esophageal squamous cell carcinoma, however its overexpression was found to inhibit cell growth. Its knockdown inhibited apoptosis by downregulating Bax and activating caspase-3 and caspase-9 (Shi, et al. 2014). SMAD 5 (SMAD family member 5) is involved in the TGF- β signaling pathway that leads to inhibition of cell proliferation. In a study, the role of SMAD5 signaling in antiproliferative effect of TGF- β in B- cell lymphoma was shown (Bakkebø, et al. 2010). As clearly seen in Table 3.4, treatment of K562 cells with fisetin also resulted in downregulation of some important genes involved in cell proliferation, invasion and adhesion for each fisetin concentration. C-KIT, proto-oncogenic cell surface receptor tyrosine kinase, regulates cell growth and proliferation, survival, and migration. Overexpression or constitutive activation of C-KIT is a common alteration in various cancer types such leukemias (Liang, et al.2013). Therefore, approaches or agents such as fisetin in our study targeting C-KIT have a potential to inhibit cancer growth. Another important common downregulated gene was MYB which is a transcription factor functioning in cell proliferation, differentiation and cell cycle events. MYB overexpression contributes to transformation in several cancer types such leukemias and several solid tumors (George and Ness 2014). Thus, its downregulation could suppress cancer cell growth like in our study. EPCAM and ICAM2 are adhesion molecules involved in cell-cell and cell-matrix adhesion. Therefore, their lower expression as compared to untreated controls could suppress leukemic cell invasion or metastasis. SERPINH1 nad SERPINB1, members of serpin superfamily of serine proteinase inhibitors, are involved in invasion and metastasis of cancer cells (Nagahara,et al. 2010). Vascular endothelial growth factor (VEGF) and its signaling are important for the induction of new blood vessel formation. Several studies indicated that haematological malignancies such as acute and chronic leukemia, lymphoma displayed increases in microvessel density (MVD) and VEGF expression in the bone marrow of patients (Medinger and Passweg 2014). Therefore, suppression of VEGF expression could block angiogenesis thus cancer cell invasion after fisetin treatment. As well as common downregulated genes, 100 μ M fisetin caused downregulation of several specific genes such as ABCC4, ABCB7, FOXK1, FOXA2 and BCL-XL (Table 3.4). ABCC4 and ABCB7 are members of the superfamily of ATP-

binding cassette (ABC) transporters and pump anticancer drugs or agents out of the cell, thus making drugs more concentrated in the cell (Zhao, et al. 2014). Higher fisetin concentration induced their downregulation. Therefore, it accumulated in the cells and became more effective for the cells. FOXK1 and FOXA2 are members of the Foxa family of forkhead/winged helix transcription factors, which have diverse roles in proliferation, invasion, development and metabolism (Hannenhalli and Kaestner 2009). Although there is no study with the exact roles of these transcription factors in cancer, they could be new targets of fisetin in K562 cells. Because, some Foxa family members have significant roles in cancer development and progression. For instance, FOXM1 is involved in progression through G1-S and G2-M phases and mitotic spindle integrity. The lower expression of FOXM1 inhibited tumor growth (Myatt and Lam 2007). BCL-XL, a member of BCL-2 family, is transmembrane molecule located in the outer membrane of mitochondria. It acts as an antiapoptotic molecule by preventing the release of cytochrome c, which causes caspase activation and then apoptosis (Lutz 2000). Its downregulation by several agents or siRNA approach has been shown to inhibit cancer cell growth and induce apoptosis like the effect of fisetin in CML cells.

A total of 1659 and 1201 genes were significantly changed ($P < 0.05$) in 100 and 150 μ M hesperetin treated K562 cells. Hesperetin treatment resulted in upregulation of some common genes for each hesperetin concentration as well as concentration specific alterations in genes (Table 3.5). DUSP1 and DUSP10 are Dual Specificity Phosphatases, which can dephosphorylate the same substrate at its both phosphotyrosine and phosphoserine/phosphothreonine parts and are involved in many signaling cellular pathways such as Mitogen Activated Protein Kinase (MAPK) pathways (Patterson, et al. 2009). In a recent study, overexpression of DUSP1 was responsible for induction of apoptosis by inhibiting p38 MAPK and NF- κ B pathways (Gil-Araujo, et al. 2014). GADD45B and GADD45G were other important upregulated genes and their roles in DNA damage, cell cycle and apoptosis were discussed above. Their upregulation could be related to hesperetin related growth suppression and apoptosis. Like fisetin treatment in K562 cells, hesperetin treatment caused upregulation of CDKN1A/p21 and CDKN2D/p19 that are involved in cell cycle arrest in response to stimuli. BCL2L11, a member of the BH3-only death activator family, was shown to protect CML cells from imatinib induced apoptosis when downregulated (Leo, et al. 2012). Therefore, its upregulation by hesperetin in this study could trigger apoptosis. PIK3IP1 (PI3K Interacting Protein 1) which binds to catalytic subunit of PI3K and reduces its activity.

Its overexpression was found to inhibit cancer development in hepatocellular carcinoma (He, et al. 2008). We concluded that hesperetin may have same effect on K562 cells. 100 μ M hesperetin also upregulated FILIP1L and CDKN1B/ p27^{Kip1}. FILIP1L (FILamin A Interacting Protein 1-Like) is a novel tumor suppressor-like protein that was downregulated in various cancers. When overexpressed, FILIP1L inhibits cancer cell invasion and metastasis through the inhibition of canonical WNT signaling (Kwon and Libutti 2014). In many human cancers, G1-checkpoint inhibitor p27 (CDKN1B) became inactive through the loss of its expression or function (Abukhdeir and Park 2008). Its upregulation could result in cell cycle arrest and then apoptosis. As seen in Table 3.5, 150 μ M hesperetin upregulated CDKN2AIP (CDKN2A interacting protein, also known as CARF), which was shown to cause growth arrest of human cancer cells through p53-p21-HDM2 pathway (Singh, et al. 2014). The other important upregulated gene after 150 μ M hesperetin treatment was NFKBIA (also known as I κ B α), a member of the NF-kappa-B inhibitor family, which inhibits NF- κ B pathway that activates the transcription of several genes involved in survival and angiogenesis (Li and Sethi 2010). Therefore, upregulation of this gene by hesperetin could suppress cell growth and induce apoptosis. Hesperetin treatment in K562 cells resulted in downregulation of several important genes as well (Table 3.6). Common downregulated genes included tubulin family members (TUBA1A, TUBAL3), STAT5A, MYC, MYB, KIT, EPCAM, ICAM2 and SERBP1 whose roles in cancer were discussed in detail throughout this part. Therefore, it is more logical to mention unique genes downregulated after hesperetin treatment. For instance, both hesperetin concentration caused downregulation of kinesins such as KIF1A and KIF11, factors involved in replication such as MCM3, POLD1 and PCNA, E2F transcription factors such as E2F2 and E2F3, factor involved in translation EIF2S2 (Table 3.6). Kinesin family members are important for cell motility, mitotic function, spindle and microtubule formation. KIF11, kinesin family member 11, encodes a motor protein controlling centrosome separation, chromosome positioning during cell mitosis (Rath and Kozielski 2012). KIF1A (Kinesin family member 1A) encodes a microtubule-dependent motor protein involved in important cellular functions such as organelle transport and cell division (Demokan, et al. 2010). Therefore, the down-regulation of kinesins by hesperetin could suggest that there is important role of hesperetin in motility of chromosomes and can be therapeutic targets in cancer. The protein encoded by MCM3 gene is one of the highly conserved mini-chromosome maintenance proteins

(MCM) that are involved in the initiation of eukaryotic genome replication. It was found to be overexpressed in various human cancers, including leukemia, lymphoma, and lung, colon and breast cancers (Ha, et al. 2004). Therefore, its downregulation by hesperetin could impair DNA replication. Another important downregulated gene was POLD1 encoding catalytic subunit of DNA polymerase delta that poses a critical role in DNA replication and repair. Therefore, its downregulation by hesperetin could inhibit the growth of CML cells through replication inhibition similar to the studies in the literature (Cao, et al. 2013). PCNA, proliferating cell nuclear antigen, is a cofactor of DNA polymerase delta. The encoded protein helps increase the processivity of leading strand synthesis during DNA replication. Therefore, like POLD1 downregulation, its downregulation could inhibit DNA synthesis, thus cell proliferation (Arivazhagan and Sorimuthu Pillai 2014). The E2F transcription factors control the cell cycle-dependent expression of genes that are essential for cellular proliferation. E2F-target genes include MYB, CDC2, cyclin E and cyclin A (Humbert, et al. 2000). Therefore, downregulation of E2F2 and E2FE could suppress proliferation of CML cells. A recent study including RNAi-related downregulation of E2F2 supported our data as well (Nakahata, et al. 2014). It is known that factors involved in translation initiation are increased and/or activated in many human cancers and could contribute to cancer development and progression (Chen, et al. 2012). Hesperetin was also found to affect protein synthesis in CML cells since it downregulated EIF2S2, eukaryotic translation initiation factor 2 subunit 2. EIF2, Eukaryotic translation initiation factor 2, functions in the early steps of protein synthesis. Both 100 and 150 μ M hesperetin also downregulated specific genes as indicated in Table 3.6. 100 μ M hesperetin affected translation related genes (EEF1G, EIF4G1), transcription related genes (POLR2B and POLR2A) and LONP1. By targeting translation initiation (EIF4G1) and elongation (EEF1G) and RNA polymerase II subunits (POLR2B and POLR2A), hesperetin could inhibit CML growth. Lon protease (LONP1) is a conserved serine peptidase playing an important role in the degradation of misfolded and damaged proteins, and supports cell viability under stress conditions. LONP1 is also a DNA-binding protein that participates in mitochondrial DNA maintenance and gene expression regulation. Therefore, changes in the expression levels of this protease gene are associated with several human diseases. Moreover, LONP1 levels are increased in different tumors and tumor cell lines. Downregulation of LONP1 in some tumor cells causes apoptosis and cell death (Bota, et al. 2005). On the other hand, 150 μ M hesperetin downregulated several MCM genes, ABCC4 (ABC

transporter discussed earlier), POLE2 (DNA polymerase epsilon subunit 2 involved in DNA replication and repair) and EIF5 (Eukaryotic translation initiation factor 5). Therefore, higher hesperetin concentration also inhibited replication and translation, thus impairing CML growth.

In addition to gene clustering, IPA tools were used to get an idea about networks with known biological pathways. These networks were found to be associated with the cell survival, proliferation, cell death, cell cycle, cell signaling pathways, adhesion, replication, transcription and translation as summarized in Tables 3.7-3.10. These results go along well with the gene clustering analysis.

In conclusion, the results of this study indicated that especially fisetin and hesperetin may have therapeutic potential in APL and CML cells due to induction of apoptosis, inhibition of cell proliferation and cell-cycle arrest. Moreover, the genetic networks identified in this study enlighten some important biological pathways that are altered by fisetin and hesperetin treatment while giving a list of candidate genes that could be targeted for CML and APL therapy. Furthermore, *in vivo* administration of most effective concentrations of fisetin and hesperetin into HL60 and K562 mice xenografts could provide a way to understand the possibility that these flavonoids may have clinical effects and might be further tested for the usage in leukemia treatment strategies.

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- Molecular Mechanisms of Drug Resistance and Its Reversal in Cancer (2014).
- Resveratrol and Quercetin Induced Apoptosis of Human 232B4 Chronic Lymphocytic Leukemia Cells by Activation of Caspase-3 and Cell Cycle Arrest (2013).
- Novel Agents Targeting Bioactive Sphingolipids for the Treatment of Cancer (2013)
- Suppression of STAT5A and STAT5B Chronic Myeloid Leukemia Cells via siRNA and Antisense-Oligonucleotide Applications with the Induction of Apoptosis (2013).
- Purification and Biochemical Characterization of an Extracellular Lipase from A Psychrotolerant *Pseudomonas Fluorescens* KE38 (2013).
- STAT Pathway in the Regulation of Zoledronic Acid-Induced Apoptosis in Chronic Myeloid Leukemia Cells (2013).
- Enalapril-Induced Apoptosis of Acute Promyelocytic Leukemia Cells Involves STAT5A (2012).
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