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Research Article

Esculetin Induces Apoptosis in Human Leukemia Kasumi-1 cells through Caspase 3 Activation

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Abstract

Objective: AML alone accounts for 20% cases of acute leukemia in children and 80% cases in adults. Among many genetic alterations, the most frequently reported translocation is between chromosomes 8 and 21, t(8; 21) or AML1-ETO. In the past decade, the anti–proliferative effect of various natural compounds including esculetin (a coumarin), has been reported in several leukemic cell lines. We have recently shown that esculetin reduces the half-life of AML1-ETO chimeric mRNA as well as mutated c-Kit transcripts in human monocytic leukemia kasumi-1 cell line harbouring the t(8;21) translocation. In the present study we established the antiproliferating activity of esculetin on kasumi-1 cell line.

Methods: In this study, cytotoxicity of the esculetin on kausmi-1 cells was investigated. The ability of esculetin to induce cell cycle arrest, alter mitochondrial potential, activate Caspase cascade and to express apoptotic markers was investigated through western blotting analysis and flow cytometry methods.

Results and conclusion: The half maximal inhibitory concentration of esculetin in kasumi-1 cells was found to be 100 µM. Ecsuletin arrested the cell cycle at G0/G1 phase in kasumi-1 cells. A significant increase in mitochondrial membrane potential and cytosolic release of cytochrome C was observed in ecsuletin treated kasumi-1 cells. This was accompanied with activation of Caspase 3 and Caspase 8 and enhanced cleavage of phospholipase C (PLC)γ-1. Annexin V apoptotic assay further corrobrated our results that esculetin mediated cytoxicity which is accompanied by cleavage of Caspase 3 is due to apoptosis in kausmi-1 cells.

Key words: Esculetin, AML1-ETO, Kasumi-1, Apoptosis, Caspase 3, Caspase 8.

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1. Introduction

Naturally occurring compounds having minimal side effects, significant abundance in nature and ease of purification are potential starting points for drug discovery. Coumarin compounds, with a typical benzopyrone framework, exemplify an important naturally occurring and synthetic oxygen-containing heterocyclins. It's derivatives readily interact with a variety of enzymes and receptors in organisms, thereby exhibiting a huge potential as medicinal drugs [1]. Esculetin is a coumarin derivative found naturally in plants including Artemisia scoparia, the leaves of limonia [2] and Ceratostigma willmottianu [3]. A plethora of pharmacological and biochemically advantageous properties have been attributed to esculetin. It inhibits cyclooxygenase and lipoxygenase enzymes, [4] and has antiadipogenic activity [5]. Esculetin mediated adipocyte apoptosis in 3T3-L1 adipocytes involves the mitochondrial pathway and has been shown to selectively alter MAPK/ ERK/ JNK pathway [6]. In addition, esculetin was shown to efficiently attenuate the oxidative stress induced cell damage via its anti-oxidant properties [7].

Earlier workers have established the neuroprotective, anti-proliferative and antitumor activities of esculetin [8]. Treatment of colon cancer cells with esculetin resulted in significant growth inhibition and G1 phase cell cycle arrest, leading to the down-regulation of expression of cyclins and cyclin-dependent kinases (CDK). This was associated with the up-regulation of p27KIP, RAS and ERK1/2 expression [9]. Anticancer activity of esculetin in benzo[a]pyrene induced lung carcinogenesis was demonstrated in mice. Further, this effect of esculetin was associated with altered Bcl-2 and NF-κB expression [10]. In another study on human oral cancer SAS cells, esculetin enhanced TRAIL-induced apoptosis primarily through the upregulation of death receptor DR5 [11].

Esculetin also inhibits the proliferation of a number of hematopoietic cell lines in a dosage-dependent manner [12-14]. In human promyelocytic leukemia HL-60 cells, esculetin induces apoptosis by increasing cytosolic translocation of cytochrome C and activation of Caspase 3 [13]. Subsequent studies revealed that esculetin inhibits the growth in HL-60 cells by cell cycle arrest in G1 phase as a result of inhibition of pRb phosphorylation [14]. The exponential cytotoxic effect of esculetin in mouse myeloma and human acute myelogenous leukemia cells under in-vitro conditions was reported recently [12]. In human leukemia U937 cells, esculetin increased the phosphorylation of ERK and JNK, thereby indicating MAPK pathway as the key regulator of esculetin induced apoptosis [15]. Further studies by the same group established that the ERK pathway participates in p21 induction, leading to decreased Cdks activity and inhibition of pRb phosphorylation in esculetin mediated G1 arrest [16]. In continuation of the study, inhibition of Bcl-2/Bax binding activity, and down-regulation of X-linked inhibitor of apoptotic protein (XIAP) expression with concomitant increase in death receptor 4 (DR4) and FasL expression was observed. Esculetin treatment also induced the degradation of β-catenin and DNA fragmentation factor 45/inhibitor of Caspase-activated DNase (DFF45/ICAD) [17]. These results indicated that esculetin-induced apoptosis in human

leukemia U937 cells was through the activation of Caspase 3 pathway [18].

Nonrandom chromosomal translocations, disrupting genes residing in the breakpoint region of the translocation, are evident in a high percentage of leukemias. Chromosomal translocation t(8;21) is found in approximately 12% of M2 type Acute Myeloid Leukemias [19, 21]. The translocation fuses the AML1/ RUNX1gene localized on chromosome 21 with the ETO/RUNX1T1/CBFA2T1gene present on chromosome 8 [22]. The kasumi-1 cell line carries the t (8;21) and have higher endogenous levels of BCL-2 [23]. The chimeric protein, AML1-ETO blocked the expression of differentiation marker GR-1 and also led to the accumulation of poly-morphonucleated cells [24]. Ras mutations are imperative in leukemogenesis and promote changes leading to transformation of cells expressing t(8;21) fusion protein. AML1-ETO also causes increased intracellular levels of reactive oxygen species (ROS) in Drosophila [25]. Downregulation of the $p27^{kip}$ and p21, cell-cycle inhibitors, is associated with AML1-ETO in leukemogenesis [26]. A negative impact on cell cycle and survival, including a G1 arrest, with decreased CDK4 and c-Myc expression is associated with AML1-ETO expression. AML1-ETO promoted apoptosis was remarkably reduced by treatment with inhibitors of JNK or coexpression of the JNK inhibitor protein 1 in inducible AML1-ETO expressing transfected U937 cells [27].

Like all AML cases, treatment of t(8;21) positive AML (M2) patients, typically begins with induction chemotherapy, which for decades has been the combination of cytarabine for seven days, together with three daily doses of an anthracycline (daunorubicin or idarubicin). If complete remission is achieved, consolidation chemotherapy is given, which in the case of t(8;21) positive AML patients consists of multiple courses of high dose cytarabine [28, 29]. Although AML patients harboring the t(8;21) translocation generally report a good prognosis and a majority of them achieve complete remission, the 5-year survival rate however is only ~50% [30]. Therefore, identification of novel therapeutic targets/drugs in t(8;21) positive AML is necessary, which will lead to better treatment options that improve patient survival. We recently reported that ecsuletin treated kasumi-1 cells harboring t(8;21) translocation show downregulation of the expression of AML1-ETO chimeric transcript with a concomitant increase in the expression of AML1 target gene expression [31]. Keeping this in mind, the current study was undertaken to check the effect of esculetin on kasumi-1 cell line with t(8;21) translocation and to explore the possible apoptotic induction by this natural coumarin. Attempts were made to understand the mechanism of apoptosis using kasumi-1 cell line as a model system.

2. Material

Propidium iodide (PI), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). FITC Active Caspase 3 Apoptosis kit was obtained from Becton Dickinson, MitoProbe™ JC-1 Assay kit was from life technologies (Eugene Oregon USA) and Annexin V Apoptosis Detection kit APC was from eBioscience (San Diego,USA). An enhanced chemiluminescence (ECL) kit was purchased from Pierce, (Rockford IL USA), RPMI 1640 medium was procured from Sigma-Aldrich and fetal bovine serum (FBS) was obtained from GIBCO-BRL (South America). Esculetin (6,7-dihydroxycoumarin, 98% purity) was

purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO as a vehicle). All other chemicals, not specifically cited here, were purchased from Sigma-Aldrich.

Antibodies

Rabbit polyclonal antibodies against PLCγ1 (PA5-17810), mouse monoclonal anti-Caspase 8 (MA1-41280) antibodies, anti-Caspase 3 active & pro (MA1-91637) antibodies and mouse monoclonal cytochrome C antibodies (MA5-11674) were purchased from Pierce Thermo (Rockford, IL USA), monoclonal antibodies against mouse βactin (sc47778) and peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were obtained from Santa Cruz, USA.

3. Methods

Cell culture

Human leukemia cell lines (RAJI and HEL) were a generous gift from Dr. Ram Ajore, (Lund University, Sweden). The Kasumi-1 cell line was a kind gift from Prof Olef Hendrich (Newcastle University, Northern Institute for Cancer Research, UK). All the cell lines were cultured in RPMI1640 medium supplemented with 15% heat-inactivated FBS, (Gibco South American origin), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (PAN Biotech) at 37 °C and 5% CO_2.

Cell viability

The cells were grown upto 70% confluence in 96 well plates and treated with the indicated concentration or time with esculetin. Control cells were supplemented with complete media containing 0.1% DMSO (vehicle control) for various time points. Stock solution (5mg/ml) of MTT was prepared in PBS and 20 µl of the stock solution was added to each well and incubated for 4 hours at 37°C. The cells were centrifuged and the supernatant was removed. DMSO was added to each well to dissolve formazan crystals. Percentage of cell viability was calculated with respect to vehicle control:DMSO (VC), provided VC was studied to be non-cytotoxic for kasumi-1 cells (data not shown). Absorbance of each well was quantified in a conventional ELISA plate reader (Tecan,Grödlg, Austria) at 570 nm.

Cell cycle

Cells were synchronized in G0 phase of cell cycle by depletion of serum for 24 hours. Synchronous population of cells was treated with 100 µM of esculetin for different time points. After the desired time interval, cells were washed with cold PBS, centrifuged and fixed overnight in 70% (v/v) ethanol at 4°C. Cells were washed twice with cold PBS and resuspended in PBS containing RNase A (0.1 mg/ml) followed by incubation for 30min at 37°C. Subsequently, 15µl of Propidium Iodide (PI) (1mg/ml) was added to the cells and incubated for 15 min in dark. The cells were then analysed for different phases of cell cycle on FACScaliber and LSR II using CellQuestPro FlowJo softwares (Becton Dickinson).

Mitochondrial Membrane Potential Assay

JC-1 is a lipophilic cationic probe which is sensitive to mitochondrial membrane potential. JC-1 is rapidly taken up by healthy polarized mitochondria where it exists as aggregates and emits red fluorescence, which is detected by FL-2 channel in flow cytometer. In a cell with injured, depolarised mitochondria, JC-1 exists as monomers in cytoplasm and does not form aggregates. JC-1 monomers emit green fluorescence, which is detected by FL-1 channel in flow cytometer. Increase in the green fluorescence reflects injured

status of mitochondria. Kasumi-1 cells were stained with JC-1 as per manufacturer's protocol. Briefly, 1 x 10^6 cells were seeded and treated with 100 µM esculetin for the desired time points. Cells were washed with PBS and resuspended in 0.5 ml of freshly prepared JC-1 working solution and incubated for 10-15 min at 37 ^{o} C in CO_2 incubator. Cells were washed twice with 1X assay buffer and resuspended in 0.5 ml of 1X assay buffer.

Active Caspase 3 staining

Expression of active Caspase 3 was quantified using FITC labeled anti Caspase 3 antibody. Cells were fixed and stained as per manufacturer's protocol. Briefly, cells were incubated with 100µM of esculetin for different time points, followed by washing with 1X PBS (twice) and were subsequently fixed in BD Cytofix/Cytoperm™ Solution (0.5ml /1x 10^6 cells). Thereafter, the cells were incubated on ice for 20 min., washed twice with 1x BD Perm/Wash™ buffer at a volume of 0.5 ml buffer/1x 10^6 cells at room temperature. The cells were incubated with antibody at room temperature for 30 min. Each test sample was washed once with 1 ml of BD Perm/Wash™ buffer (1x), and resuspended in 500 µl of BD Perm/Wash™ buffer, and analysed by flow cytometry.

Detection of apoptosis by Annexin-V FITC staining

Annexin V and Propidium Iodide (PI) staining quantitatively determines the percentage of cells actively undergoing apoptosis. The cellular staining is sensitive to the plasma membrane integrity. Annexin V has strong affinity for phosphatidylserine (PS). In apoptotic cell PS translocates from the inner membrane of the plasma membrane to the outer membrane. Annexin V binds to the exposed PS in the cells undergoing apoptosis. Viable cells with intact cell membrane exclude PI. The cells negative for both annexin V and PI are viable or are not undergoing any measurable apoptosis. Cells positive for annexin V and negative for PI are in their early phase of apoptosis as still retain cell membrane integrity. Cells positive for both Annexin V and PI are in their late phase of apoptosis or are already dead. Staining was followed as per the manufacturer's guidelines. Briefly, kasumi-1 cells were treated with 100µM esculetin for different time points. Cells were washed twice with cold PBS, and resuspended in binding buffer at a concentration of 1 X 10^6 cells/ml. APC annexin V (5 µl) and PI (5 µl) was added in each sample and incubated at RT for 15 min in dark. As per the manufacturer instruction controls were made to set up compensation and quadrants.

Western blot analysis

Esculetin (100 µM) treated cells were washed with PBS and lysed in RIPA buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 mg/ml aprotinine, 170 mg/ml leupeptin, 100 mg/ml PMSF; pH 7.5) by gentle shaking at 4^0C for 30mins. The cell lysate was centrifuged at 10,000g for 15 mins [32]. Total cytosolic protein in the clear supernatant obtained here of was estimated using BCA protein estimation kit. 30µg of protein from each sample was separated on (8-12 %) polyacrylamide gels and transferred on polyvinyldiflouride (PVDF) membrane (Millipore). Thereafter PVDF membranes were blocked and incubated with indicated primary antibodies and washed with PBS (thrice) to remove nonspecific binding. Immunoreactive bands obtained after incubation for 45 min with secondary antibody (horseradish

peroxidase (HRP) conjugated goat anti-rabbit antibody) were visualised using enhanced chemiluminescence (ECL) PLUS western blotting detection reagents.

Statistical analysis

All data is represented as mean±SD of three independent experiments. Differences between the means of control and test groups were determined using ANOVA. A value of $p<0.05$ was used as the level of significance.

4. Results and Discussion

Esculetin treatment results in inhibition of cell proliferation

Chromosomal abnormalities perturbs the hematological homeostasis and are one of the main cause of Acute myeloid Leukemia (AML), of which translocation t(8;21), t(15;17) and inversion inv(16), are most frequent [33]. Amongst these abnormalities, the translocation t(8;21) constitutes 10%-20% of the cases[34, 35]. The breakpoints have been mapped in *AML1* gene on chromosome 21q22 and *ETO* gene on chromosome 8q22 resulting in formation of the chimeric oncogene *AML1-ETO*. The translocation t(8;21) generates two fusion genes, *AML1-ETO* and *ETOAML1*, of which only the *AML1-ETO* transcript has been reported to be present in leukemic cells [36]. This transcript encodes for the fusion oncoprotein AML1-ETO that inhibits the expression of *AML1* target genes instead of activating them and thus contributes towards leukemogenesis [37]. Consequently, identification of compounds that directly or indirectly target the chimeric protein and/or transcript is an active area of research. Recent studies by Lee and co-workers have shown the anti-proliferative effect of esculetin, a natural coumarin, on U937 cells [16]. Esculetin also inhibits the growth and proliferation of HL-60 cell line and induces apoptosis [12-14] and cell cycle arrest by inhibiting phosphorylation of pRb [14]. In our previous study we established that esculetin treatment to kasumi-1 cells results in degradation of the AML1-ETO and c-Kit protein by decreasing the half life of mRNA [31].

Taking lead from previous studies, the cytotoxicity of esculetin towards three leukemic cell lines namely, kausmi-1 (AML with translocation t(8:21), HEL (erythroleukemia) and RAJI (Burkitt's lymphoma), was studied using MTT assay (Figure 1). These cells were incubated with different concentration of esculetin (50 μM to 500 μM) for different time intervals of 24, 48 and 72 hours. The results illustrate the potential of esculetin to reduce the cell number in dose and time dependent manner with lowest IC_{50} of esculetin (100 μM) in kasumi-1 cells. Since c-Kit mutations are highly prevalent in AML1-ETO patients (about 30% cases) and kasumi-1 cell line possesses both AML1-ETO t (8:21) and c-Kit activating mutation; N822K, this cell line was chosen for understanding the mechanism of esculetin action.

Esculetin-induced cell cycle arrest at G1 phase

Anticancer drugs are known to impede growth of cancerous cells by blocking the cell cycle at G0/G1, S or G2/M phase[38, 39]. Anti-proliferative plant-derived compounds like paclitaxel, silibinin, or xanthoxyletin are known to arrest cells at G2/M, G1, or S phase, respectively, and inhibit cell proliferation [40-42]. Esculetin induces G0/G1 cell cycle arrest in U937 leukemic cells [16]. To understand the mechanism of action of esculetin in kasumi-1 cells, we evaluated the effect of 100 μM esculetin on cell cycle distribution of synchronized cells (24 hours) using flow cytometry. PI staining was used to determine the cells in different phases of

cell cycle at different time intervals. An increase in the percentage of cells in G1 phase could be observed as early as 6hr post esculetin treatment which sustained upto 24 hours (Figure 2). The increase in number of cells in G1 phase was accompanied by a parallel decrease in the proliferating cells in the S phase (10%, see Figure 2 j), also compare upper panel of vehicle control with that of esculetin treated samples in lower panel). This observation suggests that esculetin inhibits the cell growth of kasumi-1 cells by arresting the cells in G0/G1 phase of cell cycle. It is pertinent to mention here that in kasumi-1 cells, in addition to cell cycle arrest, esculetin also specifically affect AML-ETO expression at transcript level (31).

Esculetin exposure leads to the loss of MMP and increase of cytochrome C.

To get further insight into the anti-proliferative effect of esculetin in kasumi-1 cells, we compared the mitochondrial membrane potential of esculetin untreated and treated cells. Mitochondrion, being the cellular powerhouse, is a lucrative target for cancer therapy. Chemotherapy has been found to induce apoptosis by targeting the mitochondria [43]. The permeability of inner mitochondrial membrane is known to get altered as a consequence of stimuli such as viral infections, free radicals, radiation and withdrawal of serum/growth factors. As a result of loss of mitochondrial transmembrane potential (ΔΨm) the release of pro-apoptotic proteins is enhanced. Moreover, the role of mitochondria as regulator of the intrinsic pathway of apoptosis is well documented [44]. Bcl-2 effect on the activity of the ion channel may also regulate apoptosis by influencing the permeability of the mitochondrial intracellular membrane and activation of Caspase 3 and PARP cleavage [15]. To evaluate the function of mitochondria in apoptosis induced by esculetin in kasumi-1 cells, change in mitochondrial membrane potential (MMP, Δψm) was analyzed post 100 μM esculetin treatment (Figure 3). The esculetin treated cells, untreated control cells as well as cells treated with carbonyl cyanide 3-chlorophenylhydrazone (as a positive control, data not shown) were stained with JC1 dye. As shown in Fig 3 h, a significant increase in MMP was observed in esculetin treated cells, which reached to 66% in 24 hours ($p<0.05$). Furthermore esculetin led to a significant increase in the cytosolic cytochrome C in a time dependent manner (Figure 4 a). These results show the direct involvement of cytochrome C and mitochondria in esculetin induced apoptosis.

Esculetin treatment induces Caspase 3, Caspase 8 and PLCγ1 in kasumi-1 cells

Cytochrome C release is known to induce Caspase 9 cleavage, which subsequently activates Caspase 8 and Caspase 3 cleavage [44]. Caspase 3 is one of the key inducer of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins, including PLCγ1 later being important for cell viability, and its cleavage facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [45]. To corroborate whether esculetin treatment to kasumi-1 cells and release of cytochrome C is associated with activation of Caspase cascade, we checked Caspase 8 and Caspase 3 activation in esculetin treated kasumi-1 cells. As shown in Fig 4 b, treatment of cells with esculetin increased the cleavage of Caspase 8. Since the mitochondrial pathway leading to release of cytochrome C and activation of Caspase 8 converge towards Caspase 3 activation, we also examined the amount

of active Caspase 3 in kasumi-1 cells using FACS. As shown in Figure 5, exposure of cells to 100 µM esculetin significantly increased active Caspase 3 at 18 hours which peaked at 48 hours ($p<0.05$). This was further substantiated with western blot analysis wherein an increase in cleaved form of Caspase 3 was obsered in cells treated with esculetin (see Figure 5g). Increased proteolytic cleavage of phospholipase C (PLC)γ-1 was also observed in a time dependent manner, in kasumi-1 cells treated with 100 µM of esculetin (Figure 4 c).

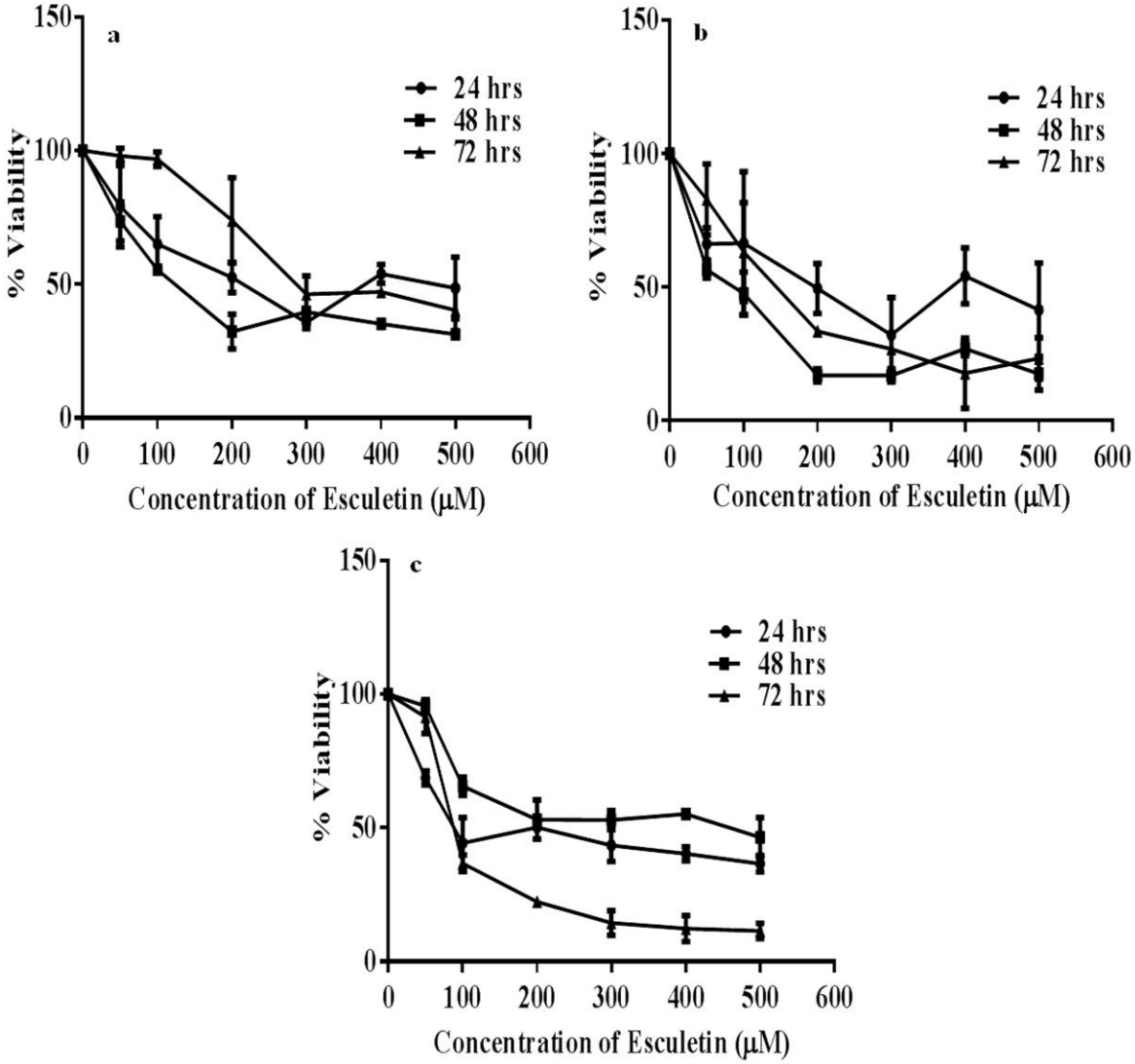


Figure 1. Effect of esculetin on cell proliferation on leukemic cell lines RAJI-1 (a), HEL (b) and kasumi-1 (c)). Cells were seeded at 2×10^5 cells/ml and were then treated with esculetin at the indicated concentrations for 24 hours, 48 hours and 72 hours. Cell viability was determined by MTT assay. The results shown are from three independent experiments and each point represents the mean±SD of three independent experiments.

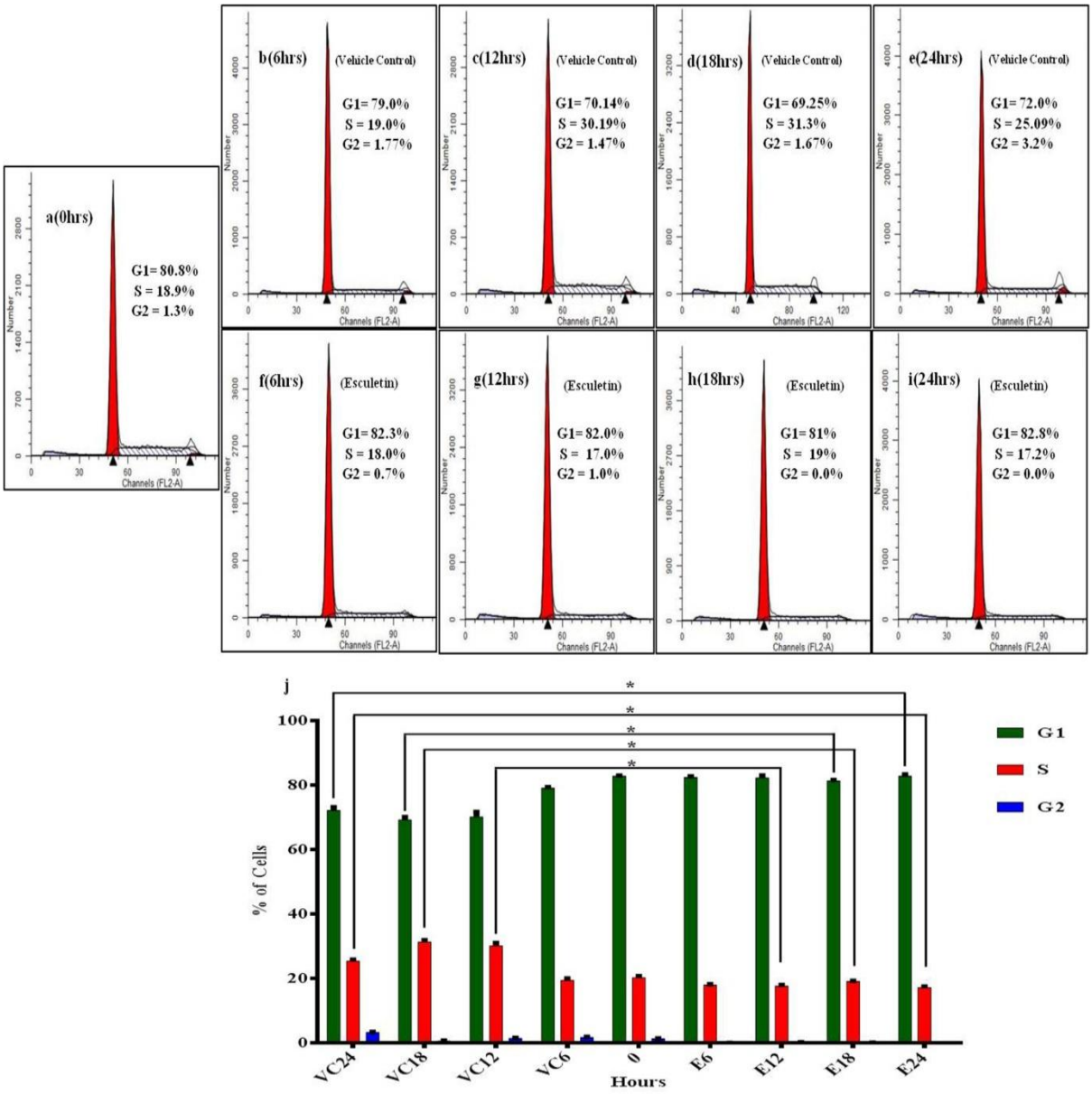


Figure 2. Cell cycle analysis of kasumi-1 cells treated with esculetin. Cells were plated at a density of 4×10^5 cells per 60-mm plate, and then incubated for 24 hours for synchronization. Next, the cells were treated with 100 µM of esculetin for the indicated times, f (6 hours), g (12 hours), h (18 hours) i (24 hours) along with (Vehicle Control: DMSO (VC)), b (6 hours), c (12 hours), d (18 hours) e (24 hours) and 0 hour synchronized cells (a), and were collected, fixed, and stained with PI for flow cytometry analysis. The percentages of cells in each phase in vehicle control and esculetin treated cells are presented (j). The data represent the average of three independent experiments. The significance was determined using ANOVA. A. (*$p<0.05$ esculetin treated vs VC).

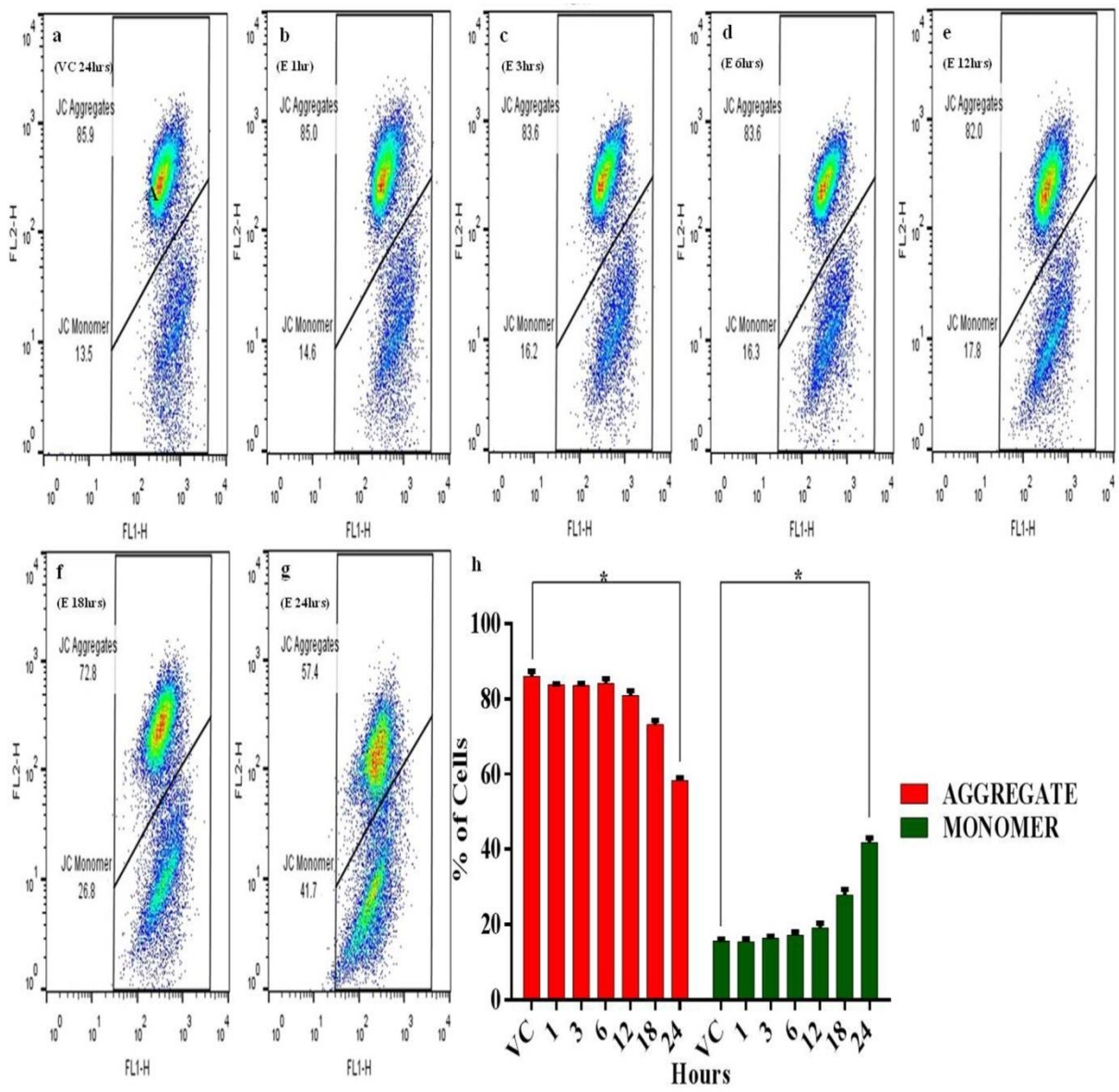


Figure 3. Effect of esculetin on the mitochondrial membrane potential of kausmi-1 cell line: Vehical control 24 hours kausmi-1 cells control (a) and cells treated with 100µM of esculetin for the different time points b (1 hour), c (3 hours), d (6 hours), e (12 hours), f (18 hours) and g (24 hours) were stained with lipophilic cationic probe JC1 and the fluorescence intensity of JC 1 dye was measured by Flow Cytometer in both FL1 (monomer) and FL2 (aggregates) channels. Cells treated with esculetin (b-f) showed a time dependent increase in fluorescence in FL-2 channel as compared to a (VC). The percentage of cells exhibiting FL-1 fluorescence and FL-2 fluorescence was plotted with respect to time (h). Each point represents the mean±SD of three independent experiments. The significance was determined using ANOVA. A (*p<0.05 esculetin treated vs VC).

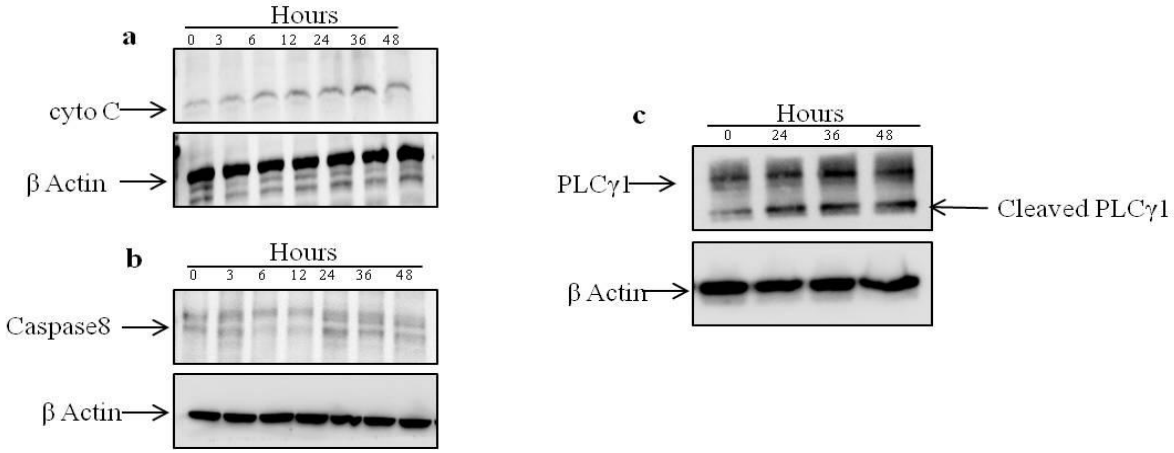


Figure 4. Expression levels of apoptosis-related proteins by esculetin treatment in Kasumi-1 cells. The cells were treated with 100 μM of esculetin for the time indicated on top of the gels (a and b). Equal amount of cell lysates (30 μg protein) were resolved on sodium dodecyl sulfate (SDS)–polyacrylamide gels, transferred to PVDF membrane, and probed with specific antibodies (anti-cytochrome C (a), anti-Caspase 8 (b) and anti-PLCγ1 (c)). β Actin was used as loading control. Results of a representative study are shown here, and two additional experiments yielded similar results.

Esculetin induces apoptosis in Kausmi-1 cell line

Apoptosis is demarcated by alteration of plasma membrane integrity as phosphatidylserine (PS) translocates from inner side of plasma membrane to the extracellular side in apoptotic cells [46]. High affinity of Annexin V for PS helps to study the early phases of apoptosis, even before the loss of cell membrane integrity [47, 48], while annexin V in combination with PI, is generally used to study the cells at later stages of apoptosis, where the cell membrane integrity is completely lost [49]. Apoptosis by esculetin in kasumi-1 cells was further corroborated by Annexin V apoptotic assay. Compared to that of DMSO treated cells, esculetin treated cells showed initiation of apoptosis within 24 hours (Figure 6 c), and by 36 hours significant number of the cells were in early apoptotic stage (Figure 6 d ($p<0.05$)). Moreover, the percentage of cells in late apoptosis stage increased in time dependent manner, with no apparent increase in the percentage of necrotic cells (Figure 6 e and f).

More than 50% of kasumi-1 cells bearing t(8;21) translocation were also shown to undergo apoptosis at concentration of 2mM oridonin, another natural plant metabolite. Interestingly, oridonin has been shown to result in caspase mediated cleavage of the chimeric AML1-ETO protein but had no effect on RNA level [50]. Similar effects were also shown with methylprednisolone and dexamethasone wherein AML1-ETO protein expression decreased in time and dose dependent manner [51]. In contrast, our previous study provide conclusive evidence that esculetin mediated decrease in chimeric protein is due to decrease in the half life of AML1-ETO mRNA [31]. Taken together,the results presented in this study suggest that the apoptotic effect of esculetin on kausmi-1 cell line is brought about by change in mitochondrial pathway leading to activation of Caspases. It will be however important to identify the target molecule(s) of esculetin so as to understand the detailed mechanism of its action.

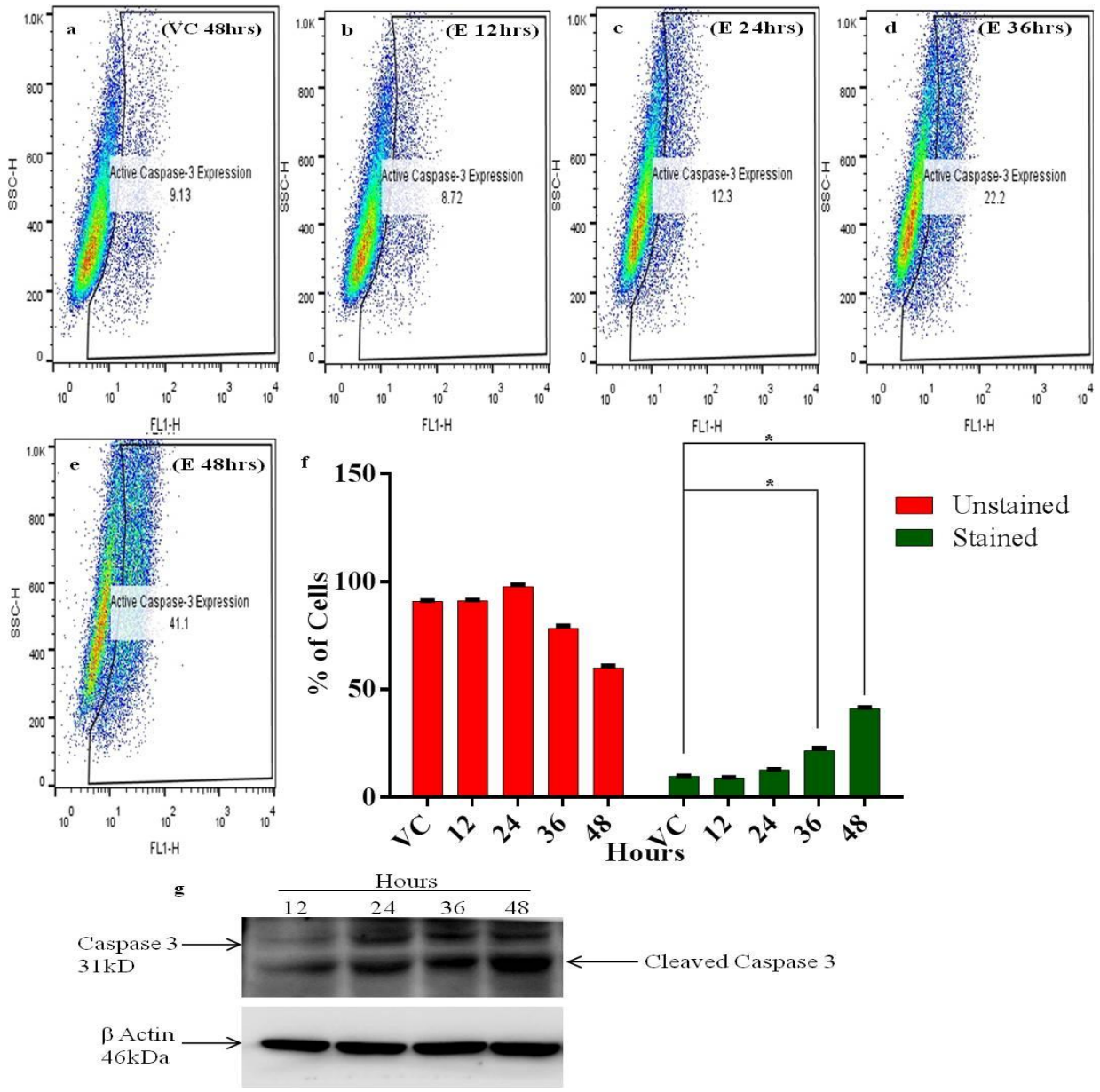


Figure 5. Activation of Caspase 3 in esculetin treated cells: Kausmi-1 cells were treated with 100 µM of esculetin for different time intervals, b (12 hours), c (24 hours), d (36 hours), e (48 hours) along with a (VC 48 hours). Cells were stained for active Caspase 3 as per the manufacturer's protocol using FITC Active Caspase 3 Apoptosis Kit obtained from BD Pharmingen. Caspase 3 activation in kasumi-1 cells was analysed by flow cytometry. Percentage of cells unstained and stained cells with FITC labelled anti-Caspase 3 antibody were plotted with respect to time (f). Each point represents the mean±SD of three independent experiments. The significance was determined using ANOVA. A (*$p<0.05$ esculetin treated vs.VC). Western blot analysis showing expression levels of normal and cleaved Caspase 3 following esculetin treatment at indicated time points (G). β Actin was used as loading control.

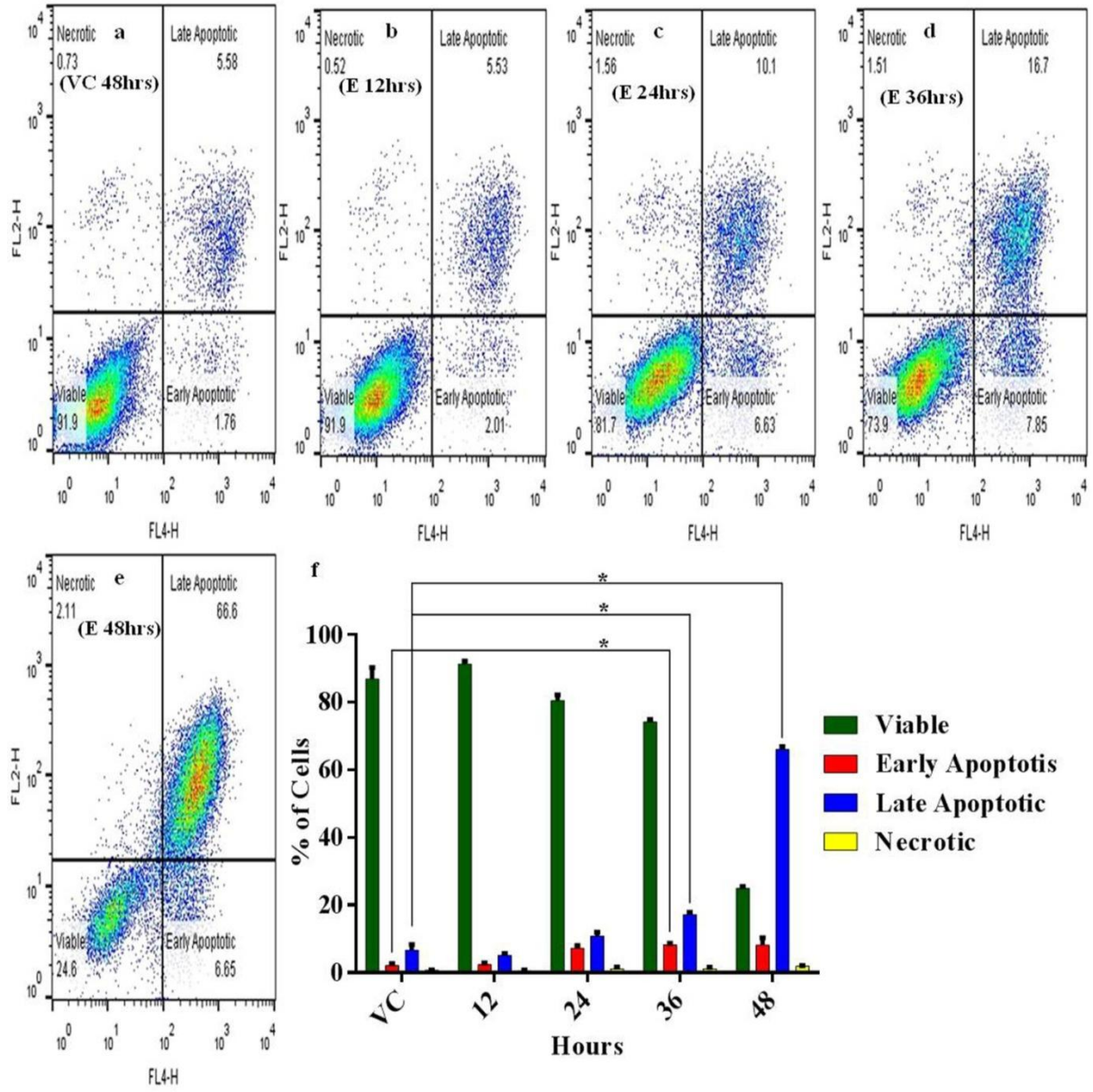


Figure 6. Esculetin induces apoptosis in kausmi-1 cell line: Kausmi-1 cells were incubated with 100 µM of esculetin for the indicated time period. Cells were stained with APC Anexin V and propidium iodide (PI) according to the manufacturer's protocol as given in APC Annexin V Apoptosis Detection Kit obtained from BD Pharmingen. Cells were analysed by Flow Cytometry. Fluorescence was measured in Both FL4-H (APC Annexin V) and FL2-H (PI). In (VC 48 hours) (a) 91.9% of cells were negative for PI and APC annexin V, indicating that cells were viable and not undergoing apoptosis. A time dependent increase in early apoptotic cells (APC Annexin V positive and PI negative) and late apoptotic cells (APC Annexin V and PI positive) was observed b (12 hours), c (24 hours), d (36 hours) and e (48 hours) as compared to that of the VC (a). The percentage of cells exhibiting fluorescence in all four panels (viable, early apoptosis, late apoptosis and necrosis) are plotted with respected to time (F). Each point represents the mean±SD of three independent experiments. The significance was determined using ANOVA. A. (*$p<0.05$ esculetin treated vs VC).

Conclusions

Acute myeloid leukemia (M2 type) is associated with AML-ETO translocation and overexpression of mutant receptor tyrosine kinase c-KIT. The antiproliferative effects of esculetin in other leukemic cells such as U937 is well studied, but the cytotoxic effects of esculetin in a cell line possessing the above two potent gene mutations was not described before, and this became the focus of the current study. This is the first report where antiproliferating affect of esculetin on kasumi-1cells possessing both AML1-ETO t(8:21) and c-Kit activating mutation; N822K) is demonstrated. Our results strongly suggest that esculetin induced cytotoxicity in Kausmi-1 cells was due cell cycle arrest and induction of apoptosis through activation of Caspase cascade. Our previous study demonstrated the ability of esculetin to effectively downregulate the gene expression of both mutant genes (AML1-ETO t(8:21) and c-Kit activating mutation; N822K) in M2 type leukemic cell line, namely kausmi-1[31]. Further studies are required to identify the targets of esculetin in kasumi-1 cells and to understand the signal transduction pathway to decipher the detailed mechanism of antiproliferative effects of esculetin.

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Authors' contributions

DS and SS conceived the study and participated in its design and coordination. SS, PA and CS carried out the Flow experiments and its analysis, SS and VC carried out the western experiment, KKA and MA participated in the design of the study. SS and DS drafted the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest

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