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ORIGINAL ARTICLE

In vitro anticancer effects of esculetin against human leukemia cell lines involves apoptotic cell death, autophagy, G0/G1 cell cycle arrest and modulation of Raf/MEK/ERK signalling pathway

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Summary

Purpose: *Esculetin is an important bioactive coumarin with amazing potential to suppress the growth of cancer cells. The present study was designed to investigate the anticancer effects of esculetin against the human leukemia HL-60 cells.*

Methods: *CCK-8 assay was used to assess cell viability. DAPI and annexin V/propidium iodide (PI) staining was performed to investigate the induction of apoptosis. Autophagy was detected by electron microscopic analysis. Flow cytometry was used for cell cycle analysis and Western blotting was used to estimate protein expression.*

Results: *Esculetin suppressed the proliferation of HL-60 cells dose-dependently. The IC_{50} of esculetin against HL-60 cells was observed to be 20 μM. The anticancer effects of esculetin against HL-60 cells occurred though different mechanisms. Esculetin induced apoptosis and autophagy in leukemia cells, which were accompanied by alteration in the expression of apoptosis as well as autophagy-related proteins. Esculetin also triggered G0/G1 cell cycle arrest in HL-60 cells, which was also accompanied by suppression of Cyclin D1 and D3. Esculetin could also block the Raf/MEK/ERK signalling pathway in leukemia cells in a concentration-dependent manner.*

Conclusion: *These results indicate that esculetin inhibits the growth of leukemia cells and hence may prove beneficial for treating leukemia.*

Key words: *leukemia, apoptosis, autophagy, esculetin, cell viability*

Introduction

Plants are rich sources of coumarins with immense pharmacological potential. Natural coumarins have gained a great deal of attention from researchers owing to their broad-spectrum biological activities [1]. Coumarins have been reported to exert anticancer effects and also possess anti-inflammatory and antibacterial properties [2]. It has been shown that coumarins inhibit the growth of cancer cells via multiple mechanisms such as activation of apoptosis, G0/G1 and G2/M phase arrest [3,4]. Esculetin is an important coumarin with enormous anticancer potential [5]. Studies have reported that esculetin induces apoptosis in human oral cancer cells via enhancement of DR5 expression [6]. In another study, esculetin has been shown to prompt apoptosis in pancreatic cancer cells by multiple mechanisms [7]. Esculetin has also been reported to inhibit the angiogenesis of cancer cells under both *in vitro* and *in vivo* conditions [8]. Nonetheless, the anticancer properties of

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esculetin have not been thoroughly investigated against human leukemia cells. Leukemia is one of the deadly cancers and according to the American Cancer Society estimates, around 0.315 million people develop a form of leukemia every year in the USA, out of which approximately 0.215 million die [9,10]. Although leukemia accounts for only 3% of all cancers, it is still one of the main causes of death due to cancer in children and persons below the age of 40 years [11]. Late diagnosis and lack of potent and safe chemotherapeutic drugs represent obstacles in the treatment of leukemia [12]. In this study, we report that esculetin inhibits the growth of human leukemia HL-60 cells via multiple mechanisms such as apoptosis, autophagy and cell cycle arrest. Owing to the therapeutic implications of the Raf/MEK/ERK signalling pathway in the treatment of cancer, a lot of research is directed at identifying molecules that can deactivate it [13]. Therefore, this study also investigated the effects of esculetin on Raf/MEK/ERK signalling pathway in leukemia cells.

Methods

Cell viability assay

The CCK-8 assay was used to determine cell viability. In brief, HL-60 cells were seeded in 96-well plates and incubated with varied concentrations of esculetin (0-640 μM) at 37°C for 24 h and subjected to treatment with 10 μl of CCK-8 solution. The cells were then subjected to incubation for 2 h at 37°C in a incubator (5% CO_2/95% O_2).OD_{450} was recorded with the help of a microplate reader.

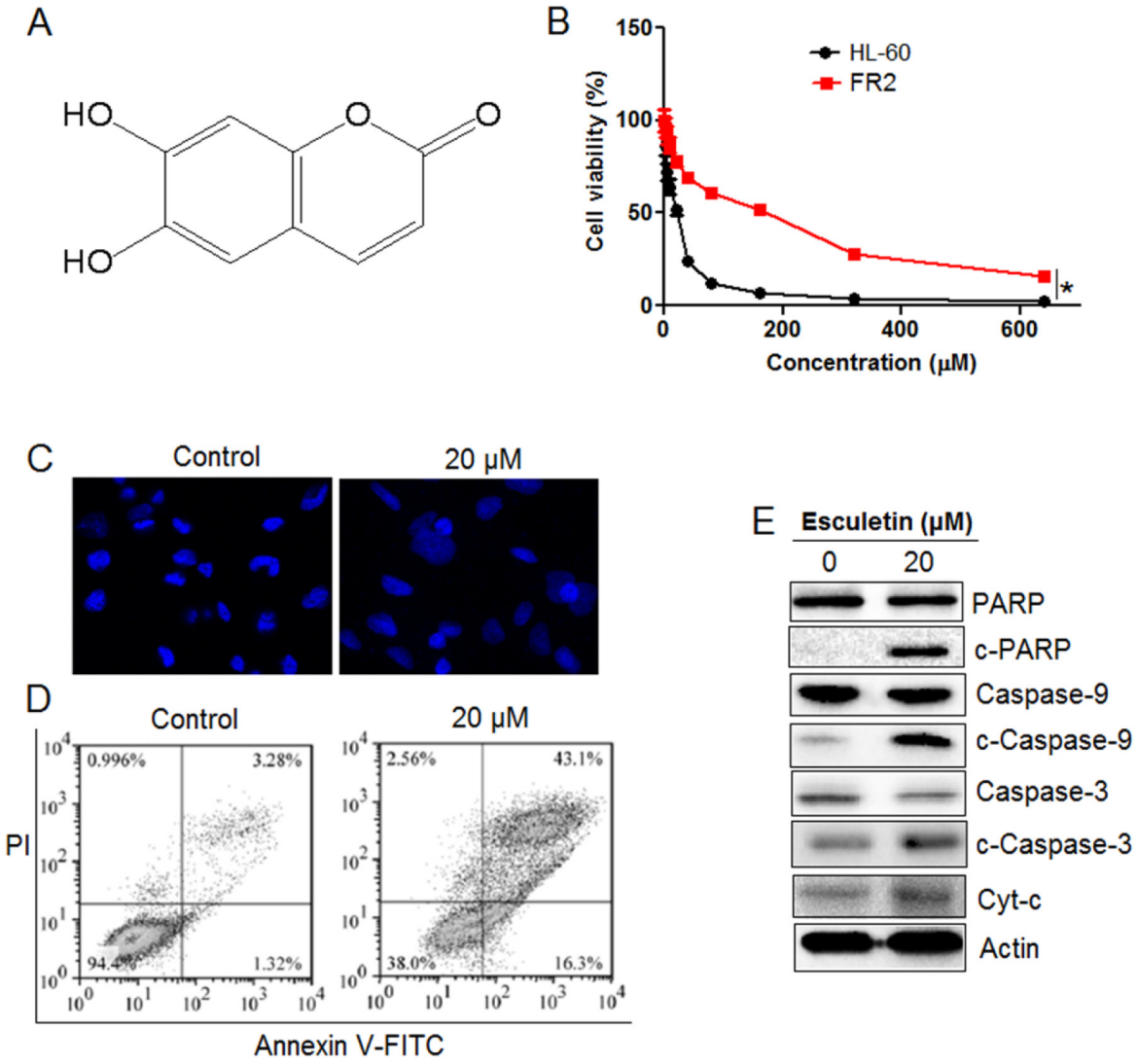

Figure 1. A: Chemical structure of esculetin. **B:** CCK-8 assay showing the viability of HL-60 and FR2 cells. Esculetin inhibited considerably the growth of HL-60 leukemia cells relative to normal FR2 cells. **C:** DAPI staining of HL-60 cells, showing that esculetin induced apoptosis in HL-60 cells. **D:** Annexin V/PI staining of HL-60 cells triggered apoptosis in HL-60 cells. **E:** Expression of apoptosis-related proteins at IC_{50} of esculetin increased the cleavage of PARP, caspases 3 and 9 and the expression of cytochrome C. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

Apoptosis assay

HL-60 cells (0.6×10^6) were seeded in 6-well plates and subjected to incubation with 0 and 20 μM of esculetin for 12 h. Following incubation, the HL-60 cells were subjected to incubation for 24 h at 37°C. As the cells sloughed off, 10 μl cell cultures were put onto glass slides and subjected to staining with DAPI. The slides were covered with a coverslip and examined under a fluorescent microscope. Annexin V/PI staining of the HL-60 cells was performed as previously described [14].

Transmission electron microscopy (TEM)

For electron microscopy, HL-60 cells were fixed in 4% glutaraldehyde 0.05 M sodium cacodylate, postfixed in 1.5% OsO4, and dehydrated in alcohol. Next, they were prepared for flat embedding in Epon 812 and then observed under a Zeiss CEM 902 electron microscope.

GFP-LC3 transfection

For the detection of autophagy, the HL-60 cells were grown to 70% confluence and transfected with GFP-LC3 plasmids using Lipofectamine 2000 as per the manufacturer's guidelines (Invitrogen, Carlsbad, California, USA). The HL-60 cells were then treated with varied concentrations of esculetin (0 and 20 μM) for 24 h and subsequently monitored by fluorescent microscopy.

Cell cycle analysis

Cell cycle analysis was performed to ascertain if esculetin can cause cell cycle arrest of HL-60 cells. In brief, the HL-60 cells were cultured and subjected to treatment with different doses of esculetin. This was followed by an overnight incubation at 37°C. The cells were then harvested by centrifugation, washed and suspended in phosphate buffered saline (PBS). Next, PI staining of the cells was performed and subsequently analyzed by FACS Calibur flow cytometer.

Western blotting

The HL-60 cells were subjected to washing with ice-cold PBS, suspended in a lysis buffer at 4°C and then shifted to 95°C. Then, the protein content of each cell extract was checked by Bradford assay. About 40 μg of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with tris-buffered saline (TBS) and exposed to primary antibodies at 4°C. After that, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualized by enhanced chemiluminescence reagent.

Statistics

Data are shown as mean ± SD. Statistical analysis was done using Student's *t*-test with GraphPad prism 7 software. P values less than 0.05 were regarded as statistically significant.

Results

Esculetin inhibited the growth of human leukemia cells via induction of apoptosis

The CCK-8 assay was used to assess the effects of esculetin (Figure 1A) on the viability of HL-60 cells. The results showed that esculetin inhibited the growth of HL-60 cells dose-dependently and exhibited an IC_{50} of 20 μM (Figure 1B). Nonetheless, the cytotoxic effects were comparatively low in normal FR2 cells (Figure 1B). DAPI staining was performed

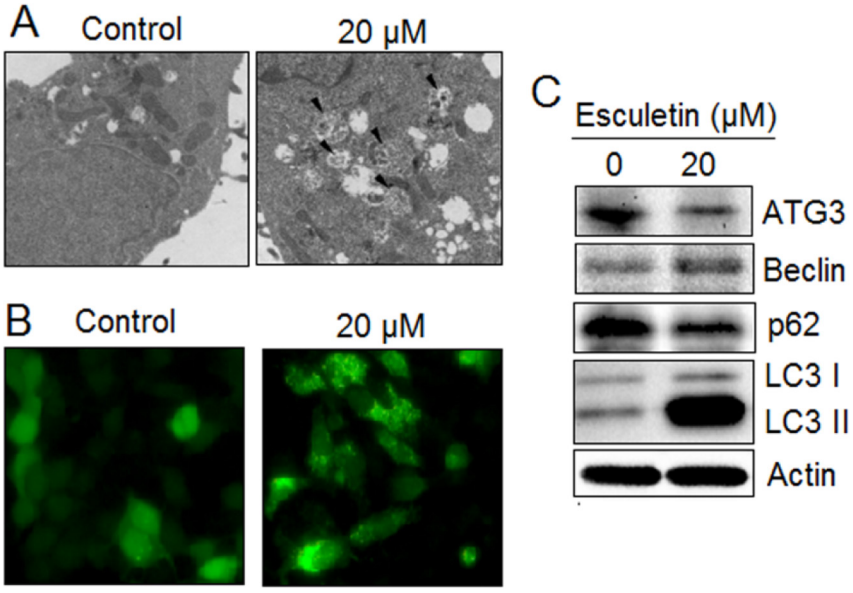

Figure 2. A: Electron microscopy of HL-60 esculetin-treated cells showing induction of autophagy by esculetin. **B:** Fluorescence microscopy showing the effect of esculetin on LC3 expression. **C:** Western blot showing the effects of esculetin on autophagy-related proteins. The experiments were performed in triplicate.

to ascertain if the anticancer effects of esculetin are due to the induction of apoptosis. DAPI staining clearly showed that DAPI-positive cells increased as the concentration of esculetin increased (Figure 1C). Moreover, annexin V/PI staining showed that the apoptotic cell percentage increased significantly upon esculetin treatment (Figure 1D). The effect of esculetin was also examined on the expression of several apoptosis-related proteins and interestingly, it was found that the expression of Bax increased, while that of Bcl-2 decreased (Figure 1E). Furthermore, esculetin enhanced the cleavage of PARP, caspase 3 and 9 and also prompted the discharge of cytochrome C in HL-60 cells.

Esculetin-induced autophagy in leukemia cells

Electron microscopic analysis was performed on esculetin-treated HL-60 cells to examine if esculetin induces autophagy in these cells. It was found that esculetin at 20 μM concentration triggered the formation of autophagic vesicles (Figure 2A). Moreover, fluorescence microscopy analysis of GFP-LC3-transfected cells showed that esculetin enhanced the expression of LC3 proteins. The Western blot analysis showed an increase in the LC3 II and Beclin expression and a decrease in the expression of ATG3 and p62 upon treatment of the HL-60 cells with esculetin (Figure 2B).

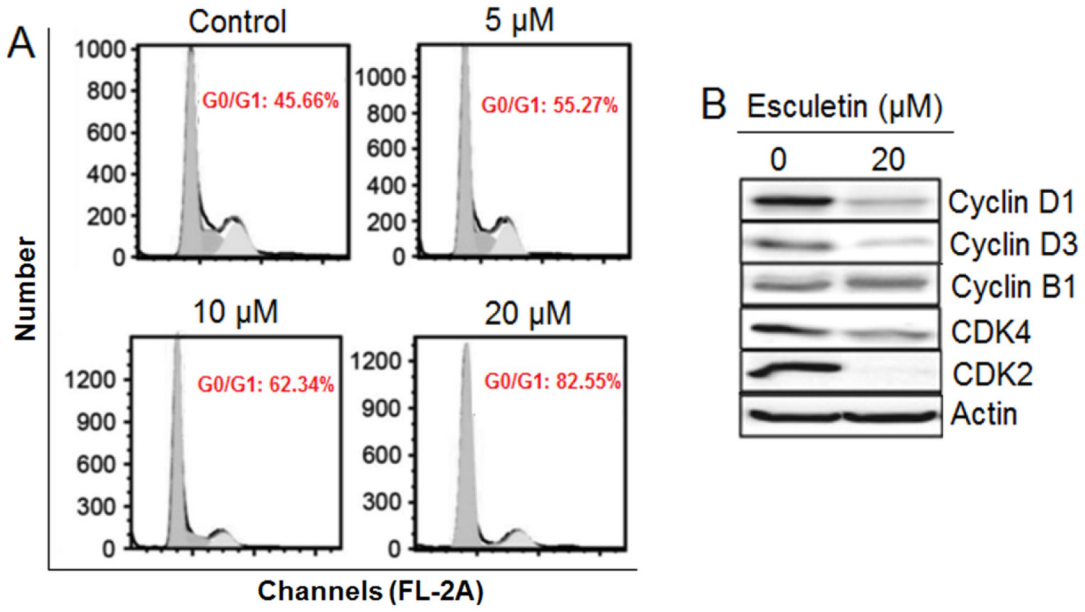

Figure 3. A: Effect of indicated concentrations of esculetin on the cell cycle distribution of HL-60 cells, showing induction of G0/G1 cell cycle arrest. **B:** Western blotting showing the effect of esculetin on the expression of cell cycle-related proteins at IC_{50}. The experiments were performed in triplicate.

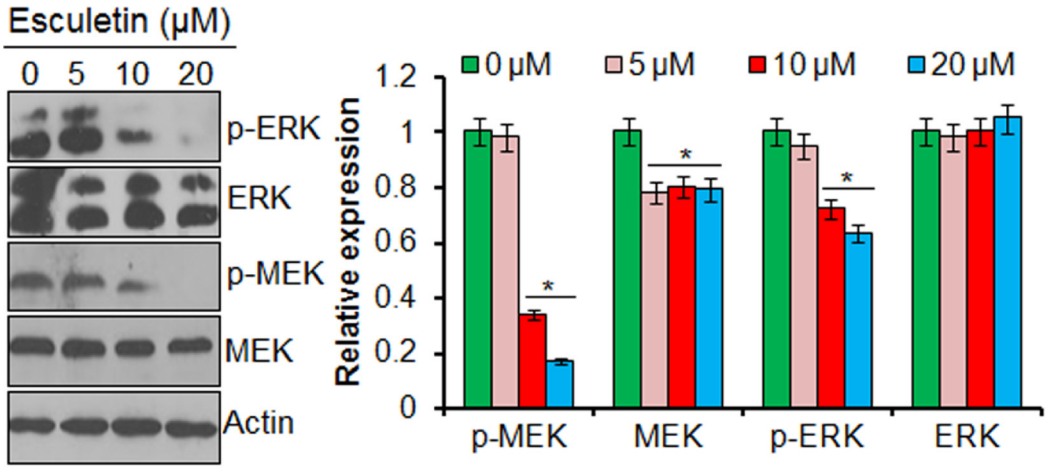

Figure 4. Western blot analysis showing the effect of esculetin on the Raf/MEK/ERK signalling pathway at indicated concentrations. As seen in the bar graph, esculetin blocked the phosphorylation of ERK and MEK in a concentration-dependent manner (*p<0.05). The experiments were performed in triplicate.

Esculetin triggered G0/G1 arrest of leukemia cells

Flow cytometry analysis of the esculetin-treated cells was carried out to find out if esculetin has any effect on the distribution of the HL-60 cells in different phases of the cell cycle. The results showed that esculetin caused significant increase in the G0/G1 phase cells, ultimately triggering G0/G1 cell cycle arrest. The percentage of the G0/G1 cells was 45.66% at the control concentration, which increased to around 82% at 40 μM concentration of esculetin (Figure 3A). The effects of esculetin were also investigated on cycle-related proteins and the results showed that esculetin suppressed the expression of cyclin D1, D3, DK4 and 2 at 20 μM concentration (Figure 3B).

Esculetin deactivated the Raf/MEK/ERK signaling pathway in leukemia cells

The Raf/MEK/ERK signaling pathway has been shown to be aberrantly activated in cancer cells. Therefore, the effects of esculetin on this pathway were also examined and showed that esculetin blocked the phosphorylation of MEK and ERK in a dose-dependent manner (Figure 4).

Discussion

Leukemia accounts for approximately 3% of all the cancer-associated mortality [15]. The chemotherapeutic agents used for the management of leukemia have a number of adverse effects, impairing us the patient quality of life [12]. Plants have served as an amazing source of anticancer agents and plant-derived molecules have shown great promise as anticancer agents [16]. In this study, the anticancer potential of esculetin, a plant-derived coumarin, was examined against HL-60 leukemia cells. The results showed that esculetin has a great potential to inhibit the growth of leukemia cells. Interestingly, the growth-inhibitory effects of esculetin were found to be cancer-specific as very low cytotoxic effects were observed against normal FR-2 cells. Previous studies have also shown the potential of esculetin. In one study, the naturally occurring coumarin esculetin has been shown to inhibit the proliferation of oral squamous cancer cells [17]. Moreover, esculetin also suppressed the growth of hepatocellular cancer cells under *in vivo* conditions [18]. DAPI staining together with Western blot analysis showed that esculetin induced apoptotic cell death of HL-60 cells by increasing the Bax/Bcl-2 ratio and triggering PARP, Caspase-3 and 9 cleavage. Moreover, esculetin also prompted the discharge of cytochrome c release. These findings are also supported by previous studies carried out on esculetin. Esculetin has been shown to suppress the survival of prostate cancer cells via activation of apoptosis [19]. This molecule has also been shown to prompt apoptosis of human malignant melanoma cells [20]. In colon cancer cells, esculetin triggered ROS-mediated apoptosis [21]. Many coumarins have also been shown to induce autophagy in cancer cells [22]. Therefore, electron microscopic analysis was also performed to ascertain if esculetin induces autophagy in HL-60 cells and it was found that it triggered the development of autophagic vesicles in these cells. Previous studies have also shown that esculetin induces cell cycle arrest of cancer cells [19] and in this study we found that esculetin induced G0/G1 cell cycle arrest of HL-60 cells, which was also accompanied by the suppression of cyclin D1, D3, CDK2 and CDK4. Raf/MEK/ERK signalling pathways have been shown to be activated in cancer cells and involved in the development and progression of several cancer types [23]. In this study, it was found that esculetin deactivates this pathway by blocking the phosphorylation of MEK and ERK.

Conclusion

The findings of this study revealed that esculetin suppresses the growth of leukemia cells via multiple modes including apoptosis, autophagy and G0/G1 cell cycle arrest. Moreover, esculetin could also deactivate the Raf/MEK/ERK signalling pathway in leukemia cells, which is indicative of its anticancer potential.

Conflict of interests

The authors declare no conflict of interests.

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