

ORIGINAL ARTICLE

Aesculetin (6,7-dihydroxycoumarin) exhibits potent and selective antitumor activity in human acute myeloid leukemia cells (THP-1) via induction of mitochondrial mediated apoptosis and cancer cell migration inhibition

Jian Gong¹, Wei-guo Zhang², Xiao-fen Feng³, Mei-juan Shao¹, Chao Xing¹

¹Medical Laboratory Center, the Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University, Wenzhou 325027, China; ²Central Laboratory, Taizhou Hospital of Zhejiang Province, Taizhou 317000, China; ³Department of Children Ophthalmology Department, Eye Optical Hospital Affiliated to Wenzhou Medical College, Wenzhou 325027, China

Summary

Purpose: The main target of the present research was to examine the antitumor properties of aesculetin in human acute myeloid leukemia cancer cells (THP-1) and peripheral blood mono-nucleated cells (PBMCs) (used as normal cell line model) along with the determination of its effects on induction of apoptosis, inhibition of cancer cell migration and changes in Bcl-2/Bax protein expressions.

Methods: MTT colorimetric bioassay was performed to study the impact of this natural compound on cytotoxicity of both cell types. Moreover, transmission electron microscopy (TEM), inverted phase contrast and fluorescence microscopic techniques were used to study the effects on cell morphology and cellular ultrastructural details connected with apoptosis. The effects of aesculetin on Bcl-2/Bax protein expressions were assessed by Western blot method.

Results: Selective and dose-dependent antiproliferative activity of aesculetin in human acute myeloid leukemia cancer cells was observed. However, the compound did not induce significant cell growth inhibition of PBMCs, which were used as normal cell controls. Fluorescence and in-

verted phase contrast microscopic techniques revealed that aesculetin led to morphological changes suggestive of apoptosis (cell shrinkage, chromatin abridgment and membrane blebbing). TEM analysis showed that aesculetin led to fragmented plasma membrane along with appearance of spherical projections (apoptotic bodies). The wound scratch widened after aesculetin treatment, indicating that aesculetin exhibits anticancer effects by suppressing the cancer cell migration. Aesculetin led to significant and dose-dependent reduction in the Bcl-2 expression while the expression of Bax was significantly enhanced resulting in overall reduction of Bcl-2/Bax ratio.

Conclusion: The results of the present work revealed that aesculetin exhibits selective anticancer effects in THP-1 human leukemia cells without causing much cytotoxicity in PBMCs. It also led to significant apoptosis induction, inhibition of cancer cell migration and decrease in Bcl-2/Bax ratio.

Key words: Aesculetin, apoptosis, cell migration, coumarin, Bcl-2, Bax

Introduction

Malignancies - either solid or not - are heterogeneous diseases characterized by uncontrolled cell division [1]. Tumors do not mean cancer, as a tumor can be benign, pre-malignant or malignant [2]. Leukemias (chronic lymphocytic leukemia and acute myeloid leukemia) represent few of the

hematological malignancies that arise from malignant transformation of different cells derived from blood, lymphatic system and bone marrow. Non-Hodgkin lymphoma is also categorized in hematological malignancies. These hematological malignancies represent a significant percentage

of cancers globally coupled with the therapeutic challenges posed by their heterogeneity and biological characteristics. Almost 10% of all malignancies in the United States are hematologic in origin. Although many treatment modalities are currently available for the treatment of leukemia, many such malignancies lack efficient treatments due to multidrug resistance [3,4]. Many risk factors and potential causes of leukemia include various chemicals, notably benzene, which are used for industrial purposes, excessive exposure to ionizing radiation, certain drugs etc [5,6]. Keeping in mind the significant upsurge in the incidence and deaths caused by leukemia in recent years, treatment of human leukemia using chemotherapeutic agents is highly anticipated. Novel treatment methodologies are essential to improve outcome. Naturally occurring compounds/extracts have always shown promise in the discovery of anticancer drugs for treating human malignancies. About half of the currently used anticancer clinical agents are either pure natural compounds or their synthetic/semi-synthetic byproducts. Many of these compounds are now being used in clinical practice, for example all-Trans Retinoic Acid. Their huge chemical diversity along with unique mode of action make these natural products effective anticancer drugs [7,8]. Natural products induce their anticancer effects via a wide range of biochemical pathways including apoptosis induction, cell cycle arrest, mitochondrial membrane potential depolarization, triggering of caspases, modulation of a variety of biochemical signalling pathway proteins which include m-TOR/PI3K/AKT signalling pathway. Many natural product-based anticancer compounds were shown to induce cell death and apoptosis in conjunction with cell cycle arrest at the G1 phase [9-11].

The primary purpose of the present work was to study the anticancer properties of aesculetin in human acute myeloid leukemia cancer cells and its effects on apoptosis induction, changes in Bcl-2/Bax ratio and cancer cell migration. Aesculetin (6,7-dihydroxycoumarin) is a naturally occurring coumarin derivative derived from the intramolecular cyclization of a cinnamic acid derivative (Figure 1).

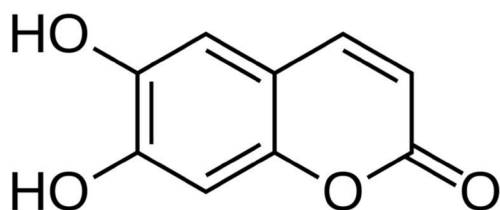


Figure 1. Chemical structure of aesculetin (6,7-dihydroxycoumarin).

Methods

Chemical and other biochemical reagents

Aesculetin ($\geq 95\%$), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The double dye acridine orange (AO)/propidium iodide (PI) were procured from Wuhan Boster Biological Technology Ltd. (Wuhan, China). RPMI-1640 and Dulbecco's modified Eagle's medium were obtained from Gibco Life Technologies, Grand Island, NY, USA. Fetal calf serum (heat-inactivated), penicillin and streptomycin were obtained from Thomas Scientific, High Hill Road, Swedesboro, USA.

Cell line and cell culture conditions

The Cancer Research Institute of Beijing, China, was kind enough to provide the THP-1 human acute myeloid leukemia cancer cell line. PBMCs were isolated from blood samples from healthy non-smoker volunteers ($n=6$). Both THP-1 and PBMC cells were maintained in Dulbecco's modified Eagle's medium accompanied with 15% FBS and 100 U/ml penicillin G, 100 $\mu\text{g/ml}$ streptomycin at 37°C in a humidified incubator.

MTT assay for cell proliferation

The cell cytotoxicity induced by aesculetin was evaluated by MTT cell viability assay using increasing doses of the drug and at different time intervals. Briefly, THP-1 cells and PBMCs were plated in 96-well plates for 18 hrs (plate density was 2×10^6 cells per well). The cells in plates were then exposed to 0, 2.5, 5, 25, 75, 100 and 200 μM aesculetin dose for 48 hrs time interval. MTT dye solution (10 μl) was then poured into each well. To solubilize MTT formazan crystals, 300 μl DMSO solution was added. Then, using an ELISA plate reader (Model 550; Bio-Rad, Hercules, CA, USA), the optical density was measured at 570 nm.

Study of aesculetin-induced apoptosis using fluorescence microscopy

The apoptosis induced by aesculetin in THP-1 cells was assessed by fluorescence microscope using the double staining dye AO/PI. The THP-1 cells were seeded in 6-well plates (cell density was 2×10^5 cells/well). Different doses of the aesculetin drug (0, 25, 75, 200 μM) were then added to the cell plates and incubated for 48 hrs. Afterwards, the drug-treated and drug-untreated cells were incubated with double dye, AO/PI (20 $\mu\text{g/ml}$ each) for 1 hr. The changes in cellular morphology were eventually seen using a fluorescence microscope (Nikon, Tokyo, Japan) under 400 x magnification.

Inverted phase contrast microscopy examination

THP-1 cells were seeded in 24-well plates at a cell density of 2×10^5 cells per well. Increasing doses of the drug (0, 25, 75, 200 μM) were added to the cell plates to examine its effects on cell morphology. DMSO 2.5% acted as the vehicle control. The cells were further in-

cubated for 48 hrs and their morphology was examined under an inverted phase contrast microscope at 200x magnification (Nikon, Tokyo, Japan).

Transmission electron microscopy (TEM) study of apoptosis

The fact that aesculetin led to apoptosis induction in human acute myeloid leukemia cancer cells was demonstrated by using TEM. In brief, the cells were seeded in a flask and were then exposed to varying concentrations (0, 25, 75, 200 μM) of aesculetin for 48 hrs. Then after harvesting and washing with PBS twice, 2.5% glutaraldehyde was added in order to make microtome sections using ultramicrotome (JEOL Co., Japan). Finally TEM investigation was done by a Transmission Electron Microscope (JEOL Co., Japan). Apoptosis evaluation was carried out by examining the appearance of apoptotic bodies and their prevalence in aesculetin-treated cells.

Wound healing assay for cell migration

THP-1 cells at a density of 2×10^5 cells per ml were seeded in a 6-well plate and left for incubation for 24 hrs to attain full confluency. The cells were starved for another 18 hrs, after which a straight cell-free wound was made using a 200 μl pipette. Each well containing cells was washed with PBS twice and then treated with 0, 25, 75, 200 μM of aesculetin for 48 hrs. After fixing, the aesculetin-treated cells were stained with 2.0% ethanol comprising of 2.5% crystal violet for 20 min. The cells were analyzed by light microscopy and the percentage of cells that drifted into the scratched area was measured.

Cell cycle analysis

For this assay, THP-1 leukemia cells were plated in 60-mm plates. The cells were then subjected to treatment with 0, 25, 75, 200 μM of aesculetin for 48 hrs. Then, the cells were trypsinized, washed with PBS twice and fixed using 70% cold ethanol for 12 hrs. After fixing, 20 $\mu\text{g}/\text{mL}$ RNase A was added and the cells were then stained with 10 $\mu\text{g}/\text{mL}$ of PI. The different cell cycle phases were eventually analyzed by flow cytometry (FACS Calibur instrument, BD Biosciences, San Jose, CA, USA).

Western blot assay

Since aesculetin targets some of the important apoptotic protein signalling pathways, we used Western blot assay. In brief, using a 16-cm plate, THP-1 cells were seeded for 24 hrs. Subsequently, the medium used was removed and substituted with new medium. The cells were then subjected to treatment with varying concentrations (0, 25, 75 and 200 μM) of aesculetin followed by 48-hr incubation. After medium removal, THP-1 cells were washed with phosphate-buffered saline solution twice before detaching cells and then lysed in RIPA buffer (Thermo Fisher Scientific Inc., Waltham, USA) and also using protease inhibitor. Subsequent to centrifugation, BCA method was used for protein content estimation. SDS-PAGE (10%) was used to separate the protein lysates (20 $\mu\text{g}/\text{lane}$) and these lysates were blotted onto nitrocellulose membranes (Millipore, MA,

USA). Each membrane was incubated with the designated primary antibodies (1:3,000 Bax and Bcl-2 specific monoclonal antibody) overnight at 4°C . The bands were observed using an ECL chemiluminescent detection kit (Perkin Elmer Cetus, Foster City, CA, USA).

Statistics

The results are expressed as mean \pm SEM, and all the experiments were done in triplicate. The statistical comparisons between different groups were done by one-way ANOVA with Tukey's posthoc tests. * $p < 0.05$, ** $p < 0.01$ were considered statistically significant.

Results

Aesculetin exhibited selective cytotoxicity against THP-1 cells

The cytotoxic effects of aesculetin on THP-1 cells were evaluated by MTT cell viability assay and the findings are shown in Figure 2. Aesculetin led to dose-dependent as well as selective cytotoxicity in THP-1 cells without causing much cell growth inhibition of PBMCs. Even at higher doses of the drug, the cytotoxic effects were marginal in PBMCs as compared to THP-1 cells. The compound showed higher IC_{50} values in PBMCs in contrast to THP-1 cells. Such a selectivity is highly required for a cytotoxic anticancer agent as it may lead to the development of safer and novel class of anticancer drugs without much serious side-effects.

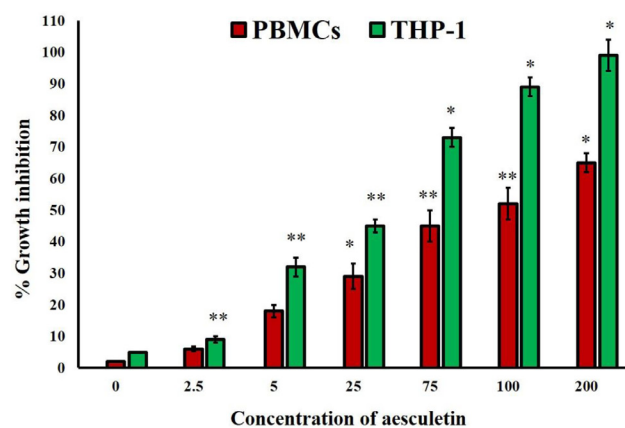


Figure 2. Graphical representation of the selective cytotoxicity induced by aesculetin in THP-1 human leukemia cells as compared to peripheral blood mono-nucleated cells (PBMCs). The cells were treated with increasing doses of the drug for 48 hrs. Data are shown as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. 0 μM (control).

Aesculetin induced apoptosis-related morphological alterations in THP-1 cells

To further confirm that aesculetin induced apoptotic morphological changes in THP-1 cells we used fluorescence microscopy using AO/PI double staining. The findings of this experiment are

depicted in Figure 3 and point out that untreated THP-1 cells displayed normal green fluorescence (green arrows in Figure 3A) while aesculetin-treated cells showed orange/red fluorescence (yellow arrows in Figure 3 B-D) suggestive of the fact that higher doses of the drug led to substantial levels of apoptosis. A dose-dependent relationship was observed between the aesculetin concentration to which the cells were subjected and the percentage of apoptotic cells.

Similar results were obtained using inverted phase contrast microscopy. Treating THP-1 cells with 0, 25, 75 and 200 μM of aesculetin led to substantial decrease of cell count along with distorted cell morphology. In contrast to the THP-1 cells without drug treatment, aesculetin-treated cells at concentrations of 25, 75 and 200 μM resulted in drastic changes in cell morphology comprising of rounding and shrinkage of THP-1 cells with disorganized layers of cells (Figure 4).

TEM ultrastructural analysis of aesculetin-induced apoptosis in THP-1 cells

To fully establish whether aesculetin encourages apoptosis in THP-1 cells, we used TEM after treating cells with the desired doses of the compound. The results are shown in Figure 5 and reveal that untreated THP-1 cells exhibited regular cellular morphology with normal chromatin and intact plasma membrane (Figure 5A). Nonetheless, treating cells with increasing doses of aesculetin led to fragmented plasma membrane along with appearance of spherical projections comprising of impaired and broken chromatin. The number of these projections (apoptotic bodies) increased with increasing dose of the compound (Figure 5 B-D). Apoptotic body formation is a crucial step involved in the apoptotic process, indicating that the anticancer effects of aesculetin are facilitated via apoptotic induction in THP-1 cells.

Aesculetin inhibited cell migration in THP-1 cells

Wound healing was performed to examine the impact of aesculetin on the cell migration capability in THP-1 cells following drug treatment. The findings of the experiment which are shown in Figure 6 indicate that in vehicle-treated control cells, the wound scratch was almost closed after 24 hrs. However, on treating cells with 75 and 200 μM of aesculetin, it was observed that the compound inhibited the wound healing in a concentration-dependent manner. The wound scratch widened after aesculetin treatment, indicating that aesculetin exhibits anticancer activity by suppressing the cancer cell migration. Drugs that can inhibit

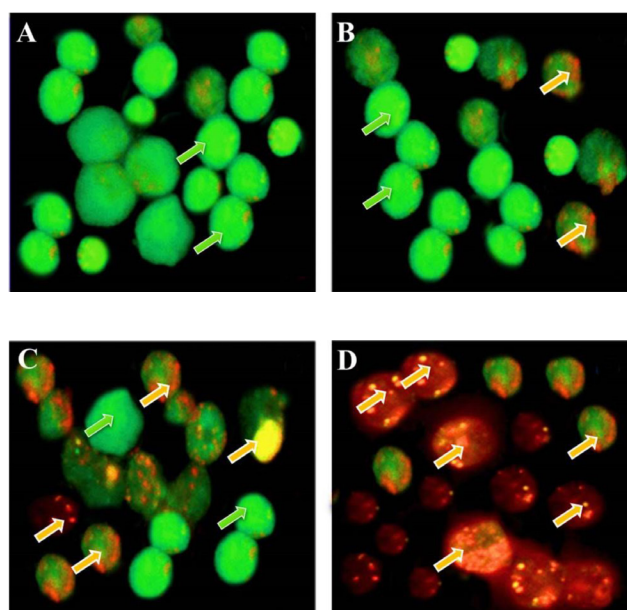


Figure 3. Fluorescence microscopic study of the apoptosis induced by aesculetin in THP-1 human leukemia cells. The cells were treated with 0 (A), 25 (B), 75 (C), and 200 μM (D) dose of aesculetin for 48 hrs and then stained with acridine orange/propidium iodide double dye and finally examined by fluorescence microscope at 400 x magnification. Green arrows indicate viable cells, while yellow arrows indicate apoptotic cells.

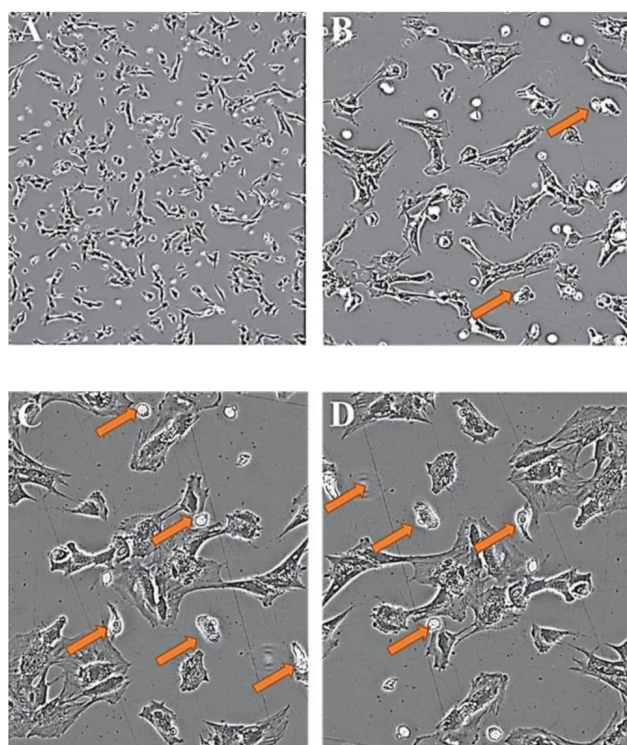


Figure 4. Aesculetin induces significant morphological changes in THP-1 human leukemia cells. The cells were treated with different concentrations 0 (A), 25 (B), 75 (C) and 200 (D) μM for 48 hrs and then observed under inverted phase contrast microscope at x200 magnification. Orange colored arrows represent cells with altered cell morphology (apoptotic cells).

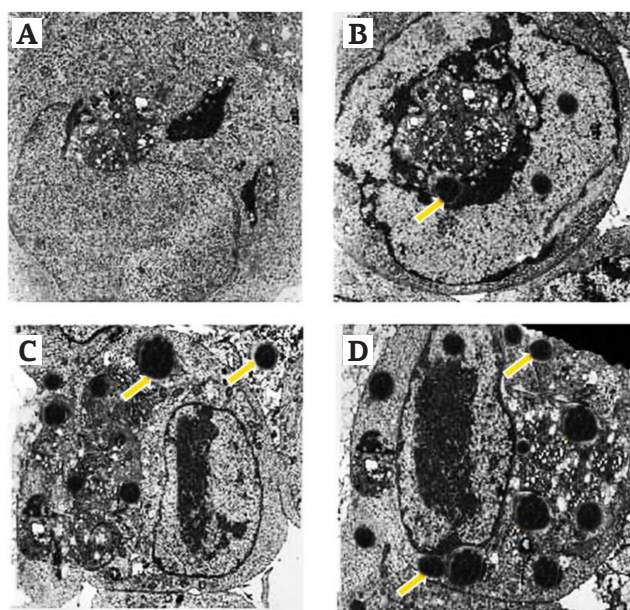


Figure 5. Aesculetin induces apoptotic cell death in THP-1 human leukemia cells. The cells were treated with 0, 25, 75 and 200 μM dose of aesculetin for 48 hrs and then analyzed using transmission electron microscope at 8000 x magnification. Arrows indicate presence of apoptotic bodies which appear as spherical projections. Note that the number of these projections increased with increasing dose of the compound.

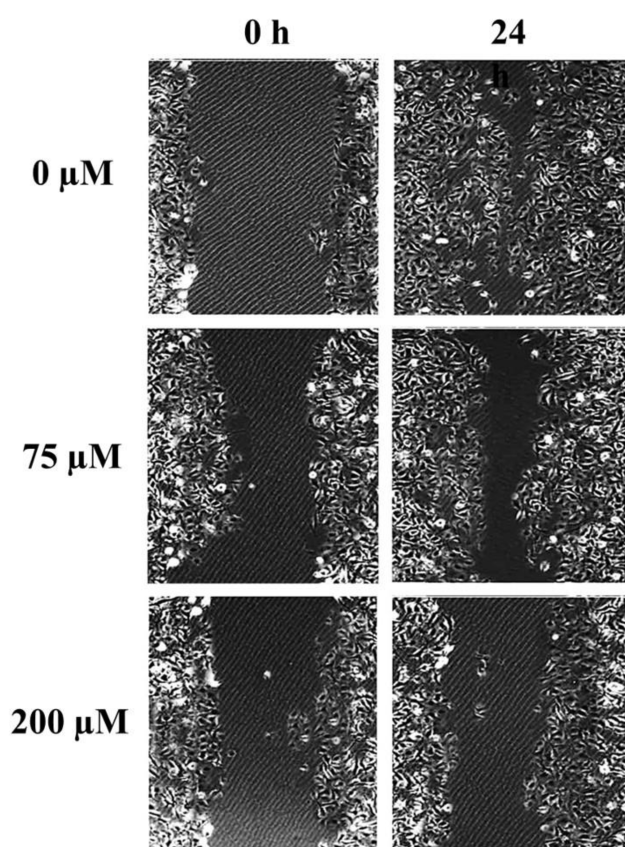


Figure 6. Aesculetin induced inhibition of cancer cell migration in THP-1 human leukemia cancer cells. Cells were treated with 0, 75 and 200 μM of aesculetin for 24 hrs. The wound was prepared by scratching with a micropipette tip.

cancer cell migration are considered to be potential anticancer agents since these compounds can lead to decreased metastasis in different cancers.

Aesculetin led to reduction of Bcl-2/Bax ratio

Our results indicated that aesculetin led to significant and dose-dependent reduction in the expression levels of Bcl-2 while Bax expression was significantly enhanced resulting in overall reduction of Bcl-2/Bax ratio. The results are shown in Figure 7.

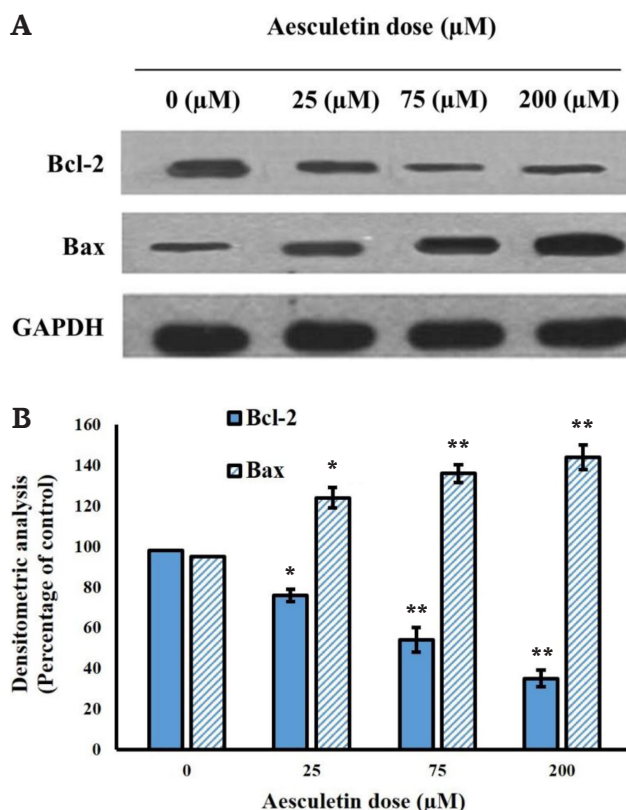


Figure 7. A: Effect of aesculetin on the expression levels of Bcl-2 and Bax which are apoptosis-related proteins. **B:** Graphical representation of the effect of aesculetin on Bcl-2/Bax ratio. Cells were treated with 0, 25, 75 and 200 μM of aesculetin for 48 hrs. * $p < 0.05$, ** $p < 0.01$ vs 0 μM (control).

Discussion

Aesculetin is a coumarin derivative isolated from chicory and many other medicinal plants in glycosidic form. Chemically it is a natural lactone which can be derived from the intramolecular cyclization of a cinnamic acid derivative. Coumarins contain a benzopyrone nucleus and on that basis coumarins are classified into many subclasses. These compounds have been shown to exhibit anticancer effects against a wide range of cancer cells [13]. Interest in the field has increased after the report of Thornes et al., who revealed the immunomodulatory and anticancer activity of cou-

marin in malignant melanoma [14]. It has been reported that coumarins led to significant inhibition of bladder cancer cells, muco-epidermoid carcinoma [15], mammary tumor cells [16] and human melanoma cells, showing promising activities for in clinical treatments. Motility and adhesive properties of neoplastic cells were affected by some of the naturally occurring coumarins. This aspect has been properly explained in the extremely invasive murine melanoma cell line B16-F10 [17]. In the current research work, we have evaluated the antiproliferative and apoptotic properties of aesculetin in THP-1 human leukemia cells in conjunction with assessment of its effects on Bcl-2/Bax ratio and cell migration. Aesculetin was previously described to show antioxidant, anti-inflammatory, anticancer and chemopreventive properties [18]. It has similarly been described to induce *in vitro* and *in vivo* antiproliferative effects in hepatocellular carcinoma cells by inducing mitochondrial-mediated apoptosis [19]. Aesculetin has also been shown to exert antitumor activity against human colon cancer cells which was mediated via the generation of reactive oxygen species and the mitochondrial pathway [20,21]. So far, no reports were found after our literature survey on the anticancer activity of aesculetin against THP-1 human leukemia cell line. In the current study we revealed selective and concentration-dependent cytotoxic effects of aesculetin in THP-1 cells. The compound did not induce significant cell growth inhibition of PBMCs, which were used as normal controls. Even at higher doses of the drug, the cytotoxic effects were marginal in PBMCs as compared to THP-1 cells. Such a selectivity is highly required for a cytotoxic anticancer agent as it may lead to the development of safer and novel class

of anticancer drugs without much serious side-effects. Additionally, fluorescence and inverted phase contrast microscopic techniques revealed that aesculetin led to morphological changes which are characteristic of apoptosis including cell shrinkage, chromatin condensation and membrane blebbing. Aesculetin-treated cells showed orange/red fluorescence suggestive of the fact that higher doses of the drug led to substantial levels of apoptosis. Similar results were obtained using inverted phase contrast microscopy. TEM analysis showed that aesculetin treatment led to fragmented plasma membrane along with the appearance of apoptotic bodies. The wound scratch widened after aesculetin treatment indicating that aesculetin exhibits anticancer effects by suppressing the cancer cell migration. Aesculetin led to significant and dose-dependent reduction in the Bcl-2 expression while the expression of Bax was significantly enhanced resulting in overall reduction of Bcl-2/Bax ratio.

In conclusion, aesculetin exhibits selective anticancer effects in THP-1 human leukemia cells without causing much cytotoxicity in PBMCs. It also led to significant apoptosis induction, inhibition of cancer cell migration and decrease in Bcl-2/Bax ratio.

Acknowledgement

This study was supported by Zhejiang Provincial Natural Science Foundation of China (Grant No. LY17H200002).

Conflict of interests

The authors declare no conflict of interests.

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