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Original investigation

Casticin: A Promising Candidate to Develop a Stem Cell Targeted Strategy in AML Treatment

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ABSTRACT

Purpose: Acute myeloid leukemia is the most common form of acute leukemia with genetic and epigenetic heterogeneity. Although current therapeutic agents provide successful remission, 5-year survival rates are still low. Insufficiency of targeting leukemia stem cells is considered as the main obstacle that causes drug resistance and relapse. Phytochemicals remain a promising source for targeted drug research. Studies showed that Casticin has antiproliferative effects on leukemic cells, but its effects on leukemic stem cells are still unclear. In this study, we aimed to investigate the antiproliferative capacity of Casticin on acute myeloid leukemia stem-like (KG1a) cells and its relatively mature parental (KG1) cells in comparison with healthy peripheral blood mononuclear cells (PBMC).

Method: The antiproliferative effects of Casticin on cells and IC50 values were determined by MTT test. The effects of Casticin on caspase 3/7 activity, apoptosis and necrosis in cells were evaluated by flow cytometry and TUNEL assays.

Results: 2 µM Casticin treatment for 24 h was increased apoptotic cell death and caspase 3/7 activation in KG1 (27.2%; 17.30%; $p<0.01$) and KG1a (21.6%; 11.35%; $p<0.01$) with relatively low necrosis (1.4% and 0.3%) compared to their control groups. TUNEL assay also confirmed Casticin-induced apoptosis in KG1 (22.3%; $p<0.05$) and KG1a cells (19.03%; $p<0.05$) compared to their control groups. Additionally, there were no significant changes in apoptosis (10.6%), caspase 3/7 activity (0.24%), necrosis (0.6%) of PBMC group compared to its control group.

Conclusion: Casticin has the capacity to induce apoptosis in both leukemia stem-like and parental leukemic cells without significantly affecting healthy cells. Therefore, we think that Casticin can be a promising compound to develop novel therapeutic strategies for final AML treatment.

Keywords: apoptosis, acute myeloid leukemia, Casticin, cancer stem cells

INTRODUCTION

Acute myeloid leukemia (AML) is the most common form of acute leukemia in children and adults with genetic, epigenetic, and phenotypic heterogeneity. Traditional chemo– and radiotherapies have restrictions related to failures in treatment and relapse. Therapeutic agents used today provide successful remission but 5-year survival rates are still quite low due to drug resistance and relapse (1, 2). The local and systemic toxicity and affecting healthy tissues are the limitations of the current therapeutics. Besides, the main obstacle in AML treatment arises from the leukemic stem cells.

Leukemic stem cells are the small and variable fraction (only 1–4%) of the total AML cells, which have the ability to self-regenerate, unlimited repopulation potential and remain in the G0/G1 quiescent phase for a long time. Therefore, these cells have the capacity to initiate leukemia and it is considered that insufficient eradication of leukemia stem cells leads to drug resistance and relapse (3, 4). Today clinicians are aware of the importance of targeting cancer stem cells, which affects therapy success. Therefore, it is important to include novel stem cell-targeted agents in therapeutic strategies (5).

For decades, several drugs for hematological malignancies have been developed from natural compounds and phytochemicals remain a promising source for targeted drug research to overcome chemoresistance and relapse due to their ability to affect specifically to cancer cells with relatively less damaging to the normal counterparts (6–8).

Apoptosis, a well-documented cell death program, plays an important role in hematopoietic system regulation. Apoptosis pathways can be initiated from the plasma membrane by death receptor ligation (extrinsic pathway) or from mitochondria (intrinsic pathway). Active caspases trigger apoptosis by two main mechanisms: intrinsic and extrinsic pathways. The response to oxidative stress and/or DNA damage promotes the intrinsic apoptotic pathway stimulation via the process of mitochondrial outer membrane permeabilization (MOMP), stimulated by pro-apoptotic proteins (Bax, Bak) and promote the release of mitochondrial cytochrome c to the cytoplasm. After the cytochrome c release, activation of the caspase cascade leads to cleavage of caspase-3 and eventually apoptotic cell death. The extrinsic pathway is stimulated by death receptors such as Fas, TNFR1 or TRAIL, that leads to receptor trimerization and cumulation of FADD or TRADD. These molecules promote the formation of death-inducing signaling complex (DISC) and stimulate caspase cascade through the activation of caspase-8. Eventually, both pathways lead to the activation of executioner caspases (caspase-3, -6 and -7), which when activated, trigger biochemical processes that initiate the disintegration of cellular components (9, 10). Escaping of the programmed cell death is an important feature of the cancer cells providing uncontrolled growth (11, 12). For this reason, triggering apoptosis is an important anti-leukemic therapeutic approach. Necrosis, the second type of cell death triggering by disease, injury, or insufficient blood supply, was originally considered as passive, accidental, unregulated cell death and might trigger inflammatory responses; hence, there is an important concern about necrosis-related inflammation (13). Studies have suggested that inflammatory microenvironment may promote drug resistance by stimulating tumor development and attenuating adaptive immune responses (14). Therefore, induction of programmed cell death is frequently considered as a more causal therapeutic approach in the treatment of malignant diseases, in contrast to non-specific necrosis.

Casticin (3′, 5-dihydroxy-3, 4′, 6, 7-tetramethoxyflavone) is a predominant component of the fruit of Vitex trifolia L. and has been widely used in traditional Chinese medicine. A body of studies have reported that Casticin induces cell death in AML cells (15–17). More importantly, research revealed that Casticin has the highest anti-proliferative effects on CCRF-CEM and CEM/ADR5000 lymphoblastic leukemia cell lines (18).

Studies have shown that Casticin has anti-leukemic activity via inducing apoptosis (15.16.18). For the ultimate AML cure, it is important to target leukemia stem cells, due to their unlimited repopulation potential that leads to drug resistance and relapse (19, 20). According to our knowledge, the effectiveness of Casticin on leukemic stem cells, which are responsible for relapse in leukemia, have not been clearly indicated yet. For the ultimate treatment of AML, targeted treatment strategies are needed that trigger programmed cell death pathways both in leukemia stem cells and leukemic blasts (20). Therefore, we aimed to evaluate the apoptotic or necrotic effect of Casticin in leukemia stem cell and relatively mature AML cells.

In this study, we investigated the capacity of Casticin to interfere the cell survival of AML stem-like cells (KG1a) and relatively mature AML cells (KG1) by MTT cell viability test, TUNEL, and flow cytometry assays. Casticin selectively induced the apoptotic cell death in both leukemia stem-like cells and parental leukemic cells without significantly affecting the healthy peripheral blood mononuclear cells (PBMC).

As the novel therapeutic strategies that can target both leukemic stem cells and leukemic blasts without damaging the healthy tissues are an unmet need, Casticin may be a candidate compound for curing AML.

MATERIALS AND METHODS

Cell culture

KG1, KG1a, and PBMC cells were obtained from ATCC. KG1, KG1a cells cultured in 20% fetal bovine serum involved Iscove's Modified Dulbecco's Medium (IMDM) (ATCC, 30–2005) at 37°C in 5% CO_2 incubator. PBMC cells cultured in 10% fetal bovine serum involved RPMI-1640 (Cegrogen, E0500–330) at 37°C in 5% CO2 incubator. Viability of cells was confirmed by using the trypan-blue exclusion. All experiments were performed 3 times in triplicate.

KG1a cell line consists of stem-like AML cells which are considered to be a valuable model for in vitro leukemic stem cell research, because they do not spontaneously differentiate to granulocyte and macrophage-like cells and not respond to colony stimulating factor. KG1 cell line, the relatively mature form of KG1a, is able to differentiate into macrophages and shows a good response to colony stimulating factor (19). PBMC cell line was used as healthy control, which is a heterogeneous population of blood cells that include macrophages, dendritic cells, monocytes, and lymphocytes.

Cell viability

The viability and IC_{50} values were determined by a colorimetric assay, which measures the reduction of 3- (4.5-dimethylthiazol-2-yl)-2.4, diphenyltetrazolium bromide (MTT) by metabolic active cells (20). Casticin (≥98% purity by HPLC) was purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; AppliChem, GmbH, Darmstadt, Germany). The concentration of the stock solution was 50 mM and further dilutions were made with fresh culture mediums. The concentration of DMSO in the final culture medium was <0.1%, which had no effect on the cell viability and it was used as vehicle in all experiments (21). Cytotoxic effects of Casticin were studied in a concentration-dependent manner (20 µM, 10 µM, 5 µM, 2 µM, 1 µM). Briefly, 50µl of cell suspension were seeded in a 96-well plate. After overnight starvation and further 24 h/48 h incubation with Casticin, 10 µl MTT solution (5 mg/mL) was added to each well and plates were incubated for 4 h. The absorbance values of the culture plates were measured at 562 nm with Biotek (ELX800, USA) microplate reader. Results were calculated with comparison of the vehicle treated control groups.

Flow cytometry

Apoptotic and/or necrotic cell death was determined by flow cytometric analysis of cells double stained with Fluorescein isothiocyanate-labeled Annexin V (Annexin V-FITC) and propidium iodide (PI) using an assay kit (BioVision, USA). Briefly, after Casticin incubation, cells were collected, washed with cold PBS, and suspended in Annexin V binding buffer. The cells were stained with Annexin V-FITC and PI for 10–15 min in the dark. After this step, the cell suspensions were analyzed flow cytometer (Navios Flow Cytometer Beckman Coulter, USA) using 488 nm excitation and bandpass filters of 530/30 nm (for FITC detection) and 585/42 nm (for PI detection) and evaluated in a flow cytometry computer program with Kaluza Analysis 2.1 Software. Cell suspensions were analyzed by counting 20.000 cells per sample with the extent of early apoptosis, late apoptosis, and necrosis determined from the percentages of bound annexin V+/PI, annexin V+/PI+, and annexin V/PI+, respectively.

Caspase 3/7 activity was determined by flow cytometric analysis of cells double stained with Caspase 3/7 Green Detection reagent (Caspase 3/7) and SYTOX AADvanced (SYTOX-ADD) dead cell stain using an assay kit (Thermofischer, CA, USA). Briefly, after Casticin incubation, cells were collected, washed with cold PBS–BSA solution and suspended in Caspase 3/7 at 37° C for 25 min in the dark. Then, SYTOX-ADD added and cells were incubated additionally 5 min at 37° C in the dark. After that, the cell suspensions were analyzed by counting 20.000 cells per sample by flow cytometer (Navios Flow Cytometer Beckman Coulter, USA) using 511 nm/533 nm (for Caspase 3/7) and 546 nm/647 nm (SYTOX-ADD) and evaluated in a flow cytometry computer program with Kaluza Analysis 2.[1] Software. Samples were analyzed with the extent of Caspase 3-7 activity (early apoptosis), late apoptosis, and necrosis determined from the percentages of bound Caspase 3–7 +/SYTOX-ADD, Caspase 3–7 +/SYTOX-ADD +, and Caspase 3–7/SYTOX-ADD+, respectively.

TUNEL Assay

Apoptosis is also confirmed by detection of DNA damage, which is the characteristic feature of late phase apoptosis. The terminal deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labelling (TUNEL) assay was performed by using using a commercial kit (Cat. no: 11684817910; Roche, Germany) according to the manufacturer's protocol. After treatment, cells were fixed, permeabilized, and incubated with the TUNEL reaction mix. After washing, incubation with conventer-POD (anti-fluorescein antibody, Fab fragment from sheep, conjugated with horse-radish peroxidase) and substrate solution (3.3′-Diaminobenzidine), cells were counterstained with hematoxylin solution. The cells were then visualized under light microscope (Olympus CKX41A, IX71) and images recorded at 100X magnification.

Statistical analysis

Cell viability data are expressed as the Mean ± SD. The data of TUNEL and flow cytometry analyses are presented as Median (± interquartile range (IQR)) and statistical analyses were performed Casticin-treated cell lines (KG1-Cast/KG1a– Cast/PBMC– Cast) against the corresponding untreated controls ((KG1-Cont/KG1a-Cont/PBMC– Cont) using the Mann–Whitney U test or 2- way ANOVA. P-value <0.05 was considered as statistically significant. Statistical analyses and graphics were performed by using SPSS version 22 and Graphpad software version 8.

RESULTS

Cell viability

It was observed that Casticin affected cell viability in KG1 and KG1a cell lines. The effect of Casticin was examined for 24 and 48 hours. Casticin decreased the cell viabilities of KG1 and KG1a at all concentrations. Particularly, 2 µM Casticin decreased cell viability to 52% -57% at 24 h but the treatment led to a dramatic decrease of cell viabilities about 9.5–11% at 48 h. Besides, PBMC cell viabilities were not decreased below 75% by Casticin treatment at all concentrations at all time periods and cell viabilities of PBMC with 2 µM Casticin treatment at 24 h and 48 h were approximately 92% and 81%, respectively (Fig. 1). According to the results, 2 µM Casticin concentration and 24 h treatment period were chosen for further analysis.

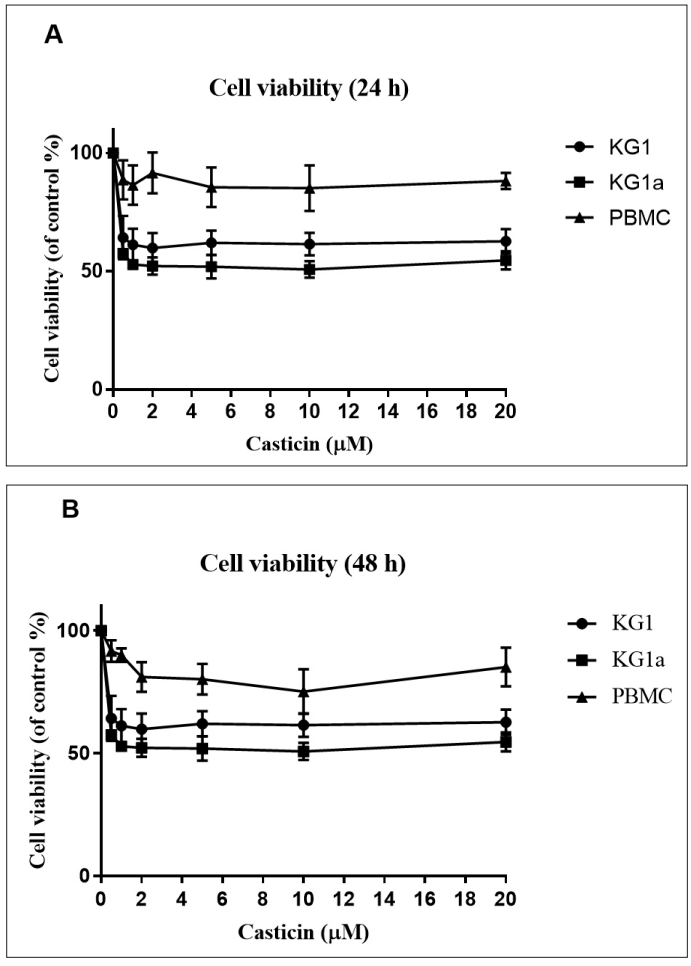

Figure 1. a. b. The relative percentage of Casticin-treated KG1, KG1a, and PBMC cell viabilities for 24 h **(a)** and 48 h **(b)**. Each point represents the relative percentage of cell viability, which was calculated according to the corresponding vehicle-treated control groups. All experiments were performed three times as triplicate and expressed as Mean ± SD.

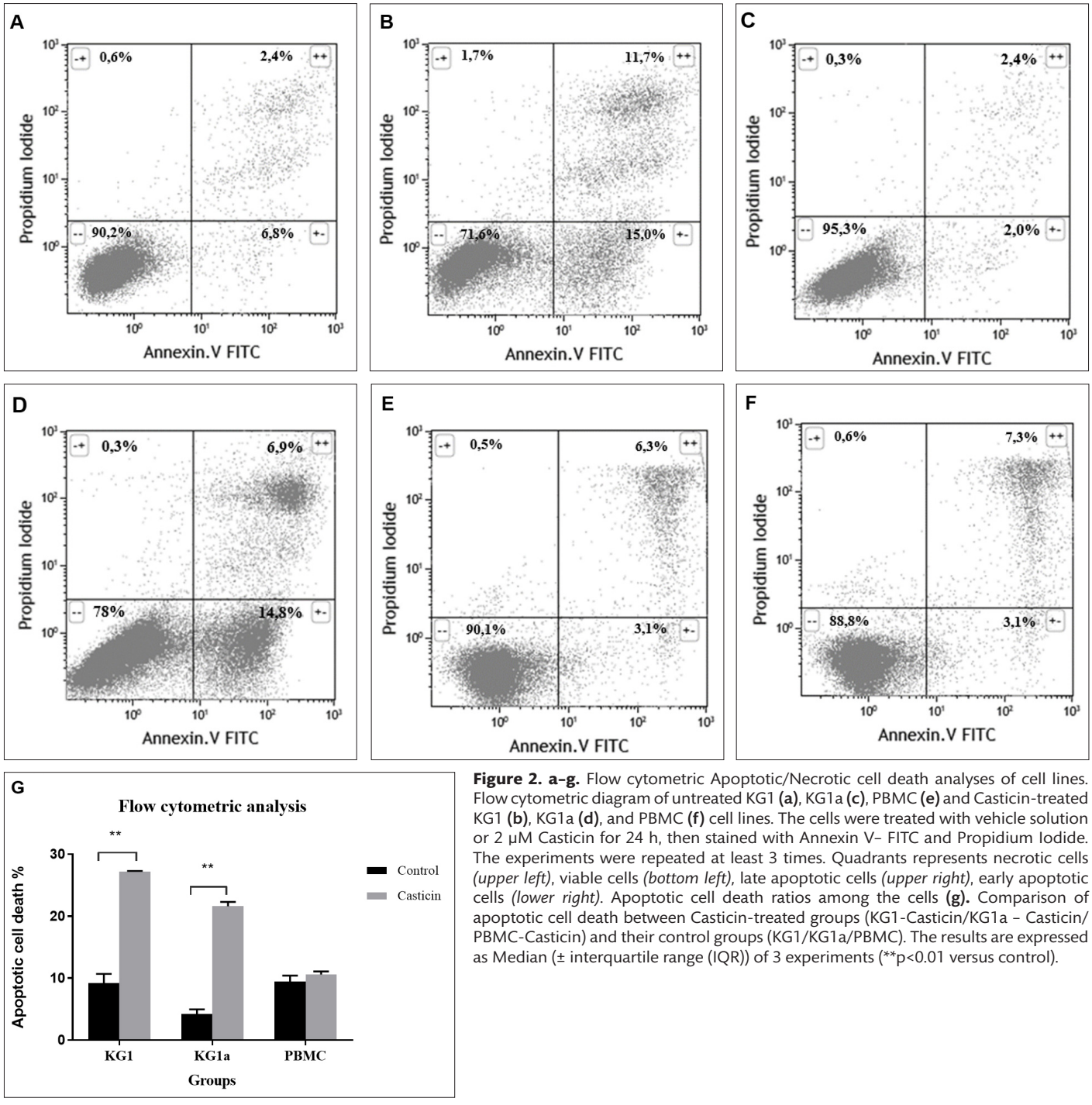

Figure 2. a–g. Flow cytometric Apoptotic/Necrotic cell death analyses of cell lines. Flow cytometric diagram of untreated KG1 **(a)**, KG1a **(c)**, PBMC **(e)** and Casticin-treated KG1 **(b)**, KG1a **(d)**, and PBMC **(f)** cell lines. The cells were treated with vehicle solution or 2 μM Casticin for 24 h, then stained with Annexin V– FITC and Propidium Iodide. The experiments were repeated at least 3 times. Quadrants represents necrotic cells *(upper left)*, viable cells *(bottom left)*, late apoptotic cells *(upper right)*, early apoptotic cells *(lower right)*. Apoptotic cell death ratios among the cells **(g).** Comparison of apoptotic cell death between Casticin-treated groups (KG1-Casticin/KG1a – Casticin/PBMC-Casticin) and their control groups (KG1/KG1a/PBMC). The results are expressed as Median (± interquartile range (IQR)) of 3 experiments (**p<0.01 versus control).

Flow cytometry

Results are expressed as the extent of apoptosis (early and late apoptosis) and necrosis. Flow cytometric analyses showed that 24 h 2 μM Casticin treatment significantly increased apoptotic cell death in KG1 (27.2%, p<0.01) and KG1a (21.7%, p<0.01) cells with relatively low necrotic cell death (1.7% and 0.3%, respectively; p>0.05) compared to their vehicle-treated control groups. These results also indicate that 24 h 2 μM Casticin treatment did not significantly induce apoptotic and necrotic cell death in PBMC cells (10.4% and 0.6%) compared to their vehicle-treated control group (p>0.05) (Fig. 2).

Results are exhibited as the percentage of caspase 3/7 activation, apoptosis and necrosis. Flow cytometric analyses showed that 24 h 2 μM Casticin treatment significantly increased the caspase 3/7 activation in KG1 (17.30%, p<0.01) and KG1a (11.35%, p<0.01) compared to their vehicle-treated control groups. Moreover, 24 h 2 μM Casticin treatment did not significantly induce the caspase 3/7 activation in PBMC cells (0.24%) compared to their vehicle-treated control group (p>0.05). Parallel to Annexin V-FITC/PI analysis, while apoptotic cell death was induced by 2 μM Casticin treatment for 24 h in KG1 (23.77%, p<0.01) and KG1a (32.96%, p<0.01) there were no significant changes in necrosis (6.23% and 5.93%, respectively;

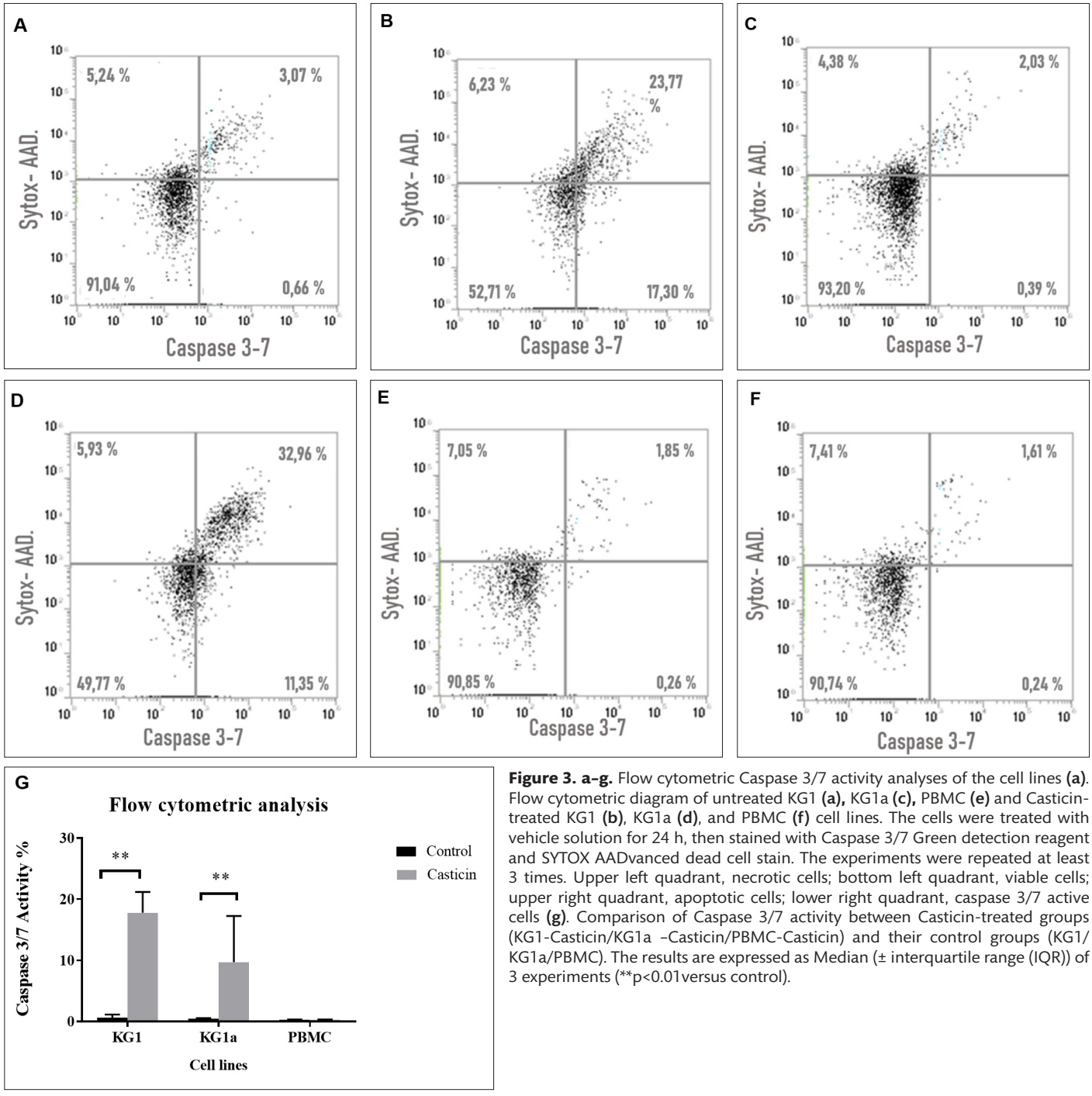

Figure 3. a–g. Flow cytometric Caspase 3/7 activity analyses of the cell lines **(a)**. Flow cytometric diagram of untreated KG1 **(a),** KG1a **(c),** PBMC **(e)** and Casticin-treated KG1 **(b)**, KG1a **(d)**, and PBMC **(f)** cell lines. The cells were treated with vehicle solution for 24 h, then stained with Caspase 3/7 Green detection reagent and SYTOX AADvanced dead cell stain. The experiments were repeated at least 3 times. Upper left quadrant, necrotic cells; bottom left quadrant, viable cells; upper right quadrant, apoptotic cells; lower right quadrant, caspase 3/7 active cells **(g)**. Comparison of Caspase 3/7 activity between Casticin-treated groups (KG1-Casticin/KG1a –Casticin/PBMC-Casticin) and their control groups (KG1/KG1a/PBMC). The results are expressed as Median (± interquartile range (IQR)) of 3 experiments (**p<0.01versus control).

p>0.05) according to their vehicle-treated control group. Also, 24 h 2 µM Casticin treatment did not significantly induce apoptotic and necrotic cell death in PBMC cells (1.61% and 7.41%) compared to their vehicle-treated control group (p>0.05) (Fig. 3).

TUNEL Assay

Results are expressed as the percentage of TUNEL-positive cells. TUNEL assays further confirmed that 24 h 2 µM Casticin treatment significantly increased apoptotic cell death in KG1 (22.3% ; p<0.05) and KG1a cells (19.03% ; p<0.05) cells compared to their vehicle-treated control groups (Fig. 4).

DISCUSSION

Acute myeloid leukemia is the most common form of acute leukemia, characterized by the accumulation of malignant myeloid precursor cells and it is called a stem cell disease due to arrested in differentiation in the bone marrow (1, 21). Conventional chemo and radiotherapies have some limitations in AML, which are arising from the stem cell characteristics and cytotoxic effects of the agents (20, 22). For more than 50 years, it has been considered that leukemic stem cells are the potential origin of AML, therefore efficiently targeting of these

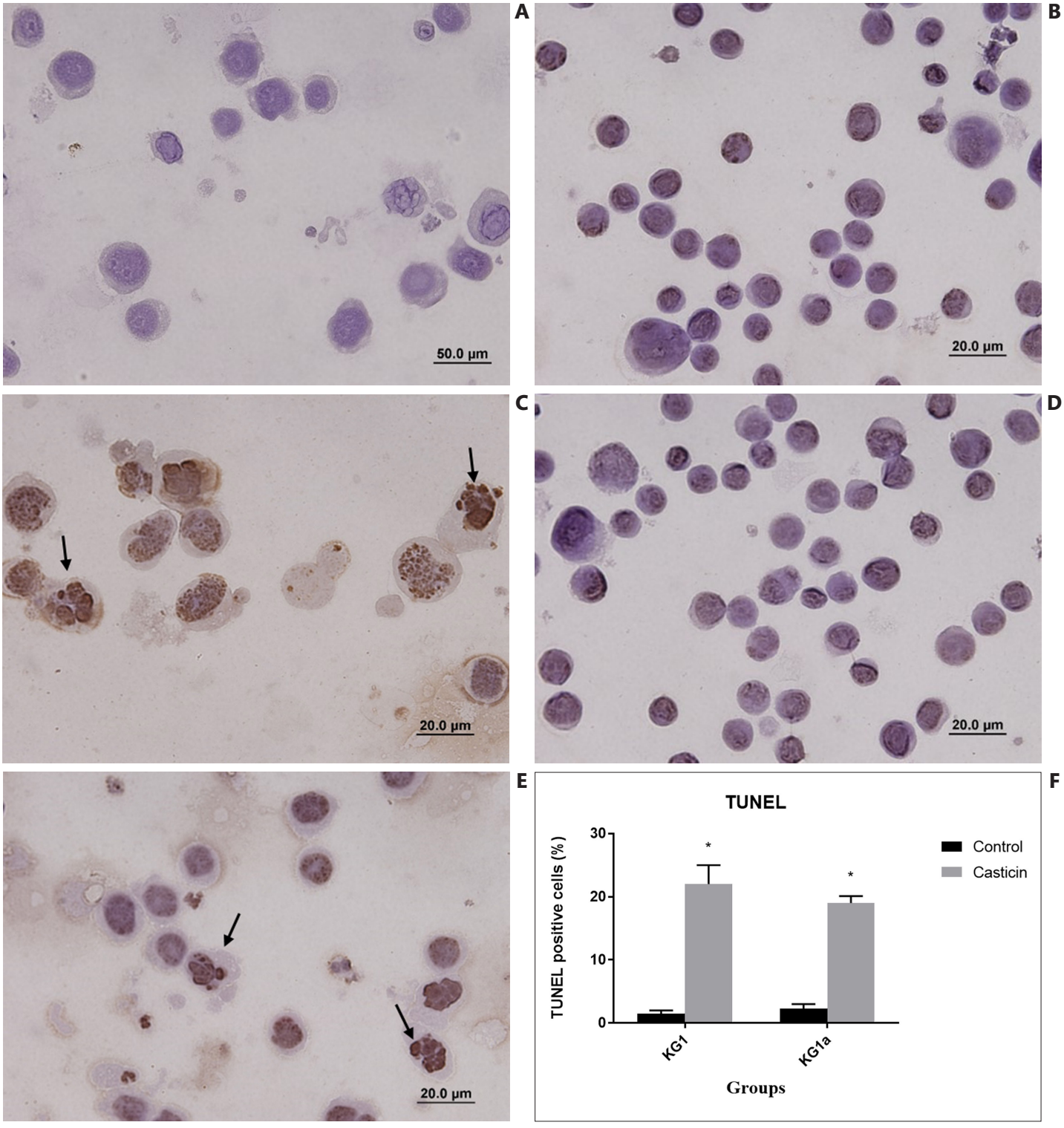

Figure 4. a–f. Apoptotic cell death analyses by TUNEL assay on negative control **(a)**, untreated control KG1 **(b)**, KG1a **(d)** and Casticin-treated KG1 **(c),** KG1a **(e)** cell lines. The cells were treated with vehicle solution or 2 µM Casticin for 24 h and stained by TUNEL assay (In Situ Cell Death Detection Kit, Roche Diagnostics) and hematoxylene. Then, photographed by light microscope at 100× magnification. The results, DNA fragmentation, **(f)** are expressed as Median (± interquartile range (IQR)) of 3 experiments (*p<0.05 versus control).

cells have a great importance in therapy success (23, 24). The less damaging features on healthy tissues while inducing cell death in cancer cells are the reasons for the preference of the natural compounds. Therefore, phytochemicals are gained attention as the promising source for the anti-leukemic drug research especially to overcome chemo-resistance and relapse (20).

Studies have reported that several polyphenols (Resveratrol, Curcumin, Quercetin, Apigenin, Chrysin, Rhein, Emodin, Aloe-Emodin, Cis-Stilbene, and Trans-Stilbene) show anti-proliferative effect in AML stem-like cells (KG1a) in a dose-dependent manner that varying 10 µM– 250 µM concentration (6–8). In addition, limited studies show that some polyphenols (such as Epigallocatechin gallate, Quercetin, Resveratrol, Curcumin) suppress cell growth in KG1 cell line also in dose-dependent manner that varying 10 µM– 100 µM concentration (25–28).

For the last decade, Casticin has been evaluated as a promising agent in leukemia treatment. For instance, Jin et al. reported that 24 h Casticin treatment induces apoptosis in acute monocytic leukemia (THP-1) and histiocytic lymphoma (U937) cell lines with IC50 values of 67.8 µM and 47.4 µM.[15] Shen et al. also showed that cell proliferation of acute myeloblastic leukemia (Kasumi-1) and acute promyelocytic leukemia (HL-60) cell lines are inhibited by 15.56 µM and 4.82 µM Casticin treatment for 48 h (16). Moreover, in 2012, Righeschi et al. reported that Casticin is the most potent polyphenol for the CCRF-CEM and CEM/ADR5000 lymphoblastic leukemia cell lines at 0.28±0.02 µM and 0.44±0.17 µM for 24 or 72 h, by stimulating apoptosis (18). Our study is consistent with Righeschi et al. and we revealed that Casticin has apoptotic cell death triggering capacity in both stem-like and mature parental cells at 2µM concentration, which is lower IC_{50} value than several polyphenols. We think that Casticin might have higher affecting potential on leukemic stem-like cells and blasts, which are important in chemo-resistance and relapse.

To obtain successful chemotherapeutics, the agent that triggers apoptosis with a low level of necrosis is preferable (11, 29). On the other hand, necrosis remains suspicious and contradictive because it may cause drug resistance by stimulating the inflammatory response (14). Despite the fact that there are limited studies have been done in AML with Casticin, we can compare our flow cytometry results, which are also confirmed by TUNEL assay, with the study of Jin et al. The researcher found that 20 µM Casticin treatment for 24 h induces apoptotic cell death in THP-1 and U937 cell lines with 29% and 24.7%, respectively (15). In this aspect, our results showed that a very low concentration of Casticin (2 µM) was significantly induced apoptosis in KG1a and KG1 cell lines with 21.7% and 27.2% for 24 h treatment. In addition, we also observed low ratios of necrosis in KG1a (0.3%) and KG1 (1.7%) cell lines with 2 µM Casticin treatment for 24 h.

Proteins from caspase family play critical roles in apoptosis, both in initiation and execution stages. Particularly, in the execution process, caspase 3 and caspase 7 can be directly cleaved and activated by caspase 9 and leads apoptotic cell death (30). Shen et al. also indicate that Casticin (5.95 µM) treatment for 48 h, promotes caspase-3 activation in chronic myeloid leukemia cells (16). Moreover, Kikuchi et al., showed that 0.3 µg/mL Casticin treatment for 12 h lead to inhibition of the proliferation via activation of caspase 8 and 9 in HL-60 cell line (17). Our analysis showed that 24 h 2 µM Casticin treatment significantly increased the caspase 3/7 activation in KG1 (17.30%, $p<0.01$) and KG1a (11.35%, $p<0.01$) compared to their vehicle-treated control groups. Moreover, 24 h 2 µM Casticin treatment did not significantly induce the caspase 3/7 activation in PBMC cells (0.24%) compared to their vehicle-treated control group ($p>0.05$). These results may provide the advantage of Casticin in development of acute anti-leukemic drugs.

Higher dosage of the chemotherapeutics, nonselective toxicity, development of therapy resistance and relapse are important limitations of the current chemotherapies in AML (22). Therefore, the therapy strategies that specifically target the leukemic stem cells and leukemic blasts without damaging healthy tissue are urgently needed. In this study, we found that Casticin suppresses the proliferation of leukemic stem– like cells and leukemic blasts and furthermore, the same concentration do not affect the healthy peripheral mononuclear cell viability. This is the first study that shows the anti-proliferative effects of Casticin in leukemia stem-like cells and leukemic blasts with minimal effects on healthy cells. Therefore, we think that Casticin is a promising candidate for the targeted treatment strategies for AML.

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Informed Consent: In vitro study

Peer-review: Externally peer-reviewed.

Ethic Committee Approval: Approval for this study was obtained from Dokuz Eylül University Non-Interventional Researches Ethics Committee. (Decision No: 2018 / 13-33, Date: 24.05.2018).

Author Contributions: Concept - TE, BS, SK; Design - TE, BS, SK; Supervision - TE, SK; Fundings - SK; Materials - SK; Data Collection and/or Processing - TE, BS; Analysis and/or Interpretation - TE, BS, HA, PK, SK; Literature Search - TE, BS, SK; Writing Manuscript - TE, BS, SK, HA, PK; Critical Review - TE, BS, SK, HA, PK

Conflict of Interest: No conflict of interest was declared by the authors.

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