Evaluation of phytoestrogens in inducing cell death mediated by decreasing Annexin A1 in Annexin A1-knockdown leukemia cells

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Abstract

Background Phytoestrogens are plant compounds that are structurally similar to estrogen and that possess anti-cancer properties. Previous studies have reported that coumestrol, daidzein and genistein could induce cell death by reducing Annexin A1 protein in leukemic cell lines. Annexin A1 (ANXA1) is involved in cell progression, metastasis, and apoptosis in several types of cancer cells. The present study sought to investigate if the effects of phytoestrogens on apoptosis, cell cycle arrest and phagocytosis in ANXA1-knockdown leukemic cells are mediated through ANXA1 or occurred independently.

Methods Transfection of ANXA1 siRNA was conducted to downregulate ANXA1 expression in Jurkat, K562 and U937 cells. Apoptosis and cell cycle assays were conducted using flow cytometry. Western blot was performed to evaluate ANXA1, caspases and Bcl-2 proteins expression. Phagocytosis was determined using hematoxylin and eosin staining.

Results The expression of ANXA1 after the knockdown was significantly downregulated in all cell lines. Genistein significantly induced apoptosis associated with an upregulation of procaspase-3, -9, and -1 in Jurkat cells. The Bcl-2 expression showed no significant difference in Jurkat, K562 and U937 cells. Treatment with phytoestrogens increased procaspase-1 expression in Jurkat and U937 cells while no changes were detected in K562 cells. Flow cytometry analysis demonstrated that after ANXA1 knockdown, coumestrol and genistein caused cell cycle arrest at G2/M phase in selected type of cells. The percentage of phagocytosis and phagocytosis index increased after the treatment with phytoestrogens in all cell lines.

Conclusion Phytoestrogens induced cell death in ANXA1-knockdown leukemia cells, mediated by Annexin A1 proteins.

Keywords Annexin A1 · siRNA · Apoptosis · Caspase cascade · Cell cycle arrest · Phagocytosis · Human leukemic cell lines

Abbreviations

ANXA1	annexinA1
Annexin V-FITC	annexin V-fluorescein
PI	propidium iodide

RPMI-1640	Roswell Park Memorial Institute medium 1640
IMDM	Iscove's modified Dulbecco's medium
FBS	fetal bovine serum
siRNA	small interfering RNA
ER	estrogen receptor

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Introduction

Phytoestrogens are plant-derived compounds that have anti-cancer properties, including promoting cell death, inhibiting angiogenesis and cell growth, and causing cell cycle arrest [1]. Phytoestrogens can be classified into four major groups: lignan, isoflavones, stilbenes and coumestans. Coumestrol is classified as coumestans while daidzein and genistein are

classified as isoflavon [2, 3]. Previous studies showed that coumestrol induced apoptosis in breast cancer cells (MCF-7), prostate cancer cells (PC3), and colon cancer cells (HCT116) through different mechanisms of apoptosis [4–6]. Daidzein induces apoptosis in breast cancer cells, MCF-7 and MDA-MB-453, and human preadipocytes cells, AML-1 [7, 8] while genistein induces apoptosis in stomach, prostate, pancreatic carcinoma cells and other cancer cells [9–11]. Apoptosis is a mechanism of cell death with morphological characteristics of cell shrinkage and membrane blebbing triggered by various death stimuli, including stress, heat, and UV radiation [12, 13]. Two major apoptosis pathways are extrinsic, involving binding of ligands to cell surface ‘death receptors’, and intrinsic, involving mitochondria [14]. Apoptosis involves the activation of caspases, a family of cysteine-protease proteins and Bcl-2 family [15]. The caspases family can be divided into two categories: initiator and effector caspases. The activation of initiator caspases, such as caspase -2, -8, -9 and -10, through the cleavage of aspartate (Asp) residue triggers the execution of effector caspases, such as caspase -3, -6 and -7. Meanwhile, Bcl-2 is an anti-apoptotic protein that maintains mitochondrial membrane potential (MMP) [16, 17].

Coumestrol inhibits cell growth in human prostate cancer cells, PC3 and LNCaP [18]. In breast cancer cells (MCF-7) and colon cancer cells (HCT116), coumestrol inhibits cell growth by increasing ROS production, and p53 and p21 activation [6]. Daidzein inhibits cell cycle progression in breast cancer cells (MCF-7 and MDA-MB-453). It causes cell cycle arrest at G2/M phase by decreasing cell cycle distribution at G0/G1 phase via downregulation of cyclin D, CDK2 and CDK4 expressions in both cells [8]. Genistein also induces G2/M arrest in cervical cancer cells (ME180), prostate cancer cells (PC3), and breast cancer cells including MCF-7, MDA-MB-231 and MDA-MB-468 [19]. Cell cycle is a sequence of events that manages the growth and reproduction of new cells by regulating specific proteins. Cells respond to DNA damage or death stimuli by halting the progression of cell cycle or by undergoing programmed cell death. Dysregulation of a cell cycle is the main reason for uncontrollable cell growth and eventually causes cancer [20–22].

Recently, a study demonstrated that phytoestrogens induced apoptosis, cell cycle arrest and phagocytosis activity and decreased ANXA1 expressions in leukemic cells (*unpublished data*). Annexin A1, an endogenous glucocorticoid-regulated protein was first discovered to be involved in the inflammatory processes [23]. It has two domains: the C-terminal domain is for Ca^{2+} and membrane binding sites while the N-terminal domain is for proteolysis and phosphorylation involving a broad range of biological processes in various cancer cells

[24]. The ANXA1 activates a cascade of signaling pathways through an association with formyl peptide receptors (FPRs). Previous studies reported that ANXA1 was involved in different cell processes such as cell proliferation, migration, differentiation and apoptosis in various cancer cells [25, 26]. However, little is known about phytoestrogens associated with Annexin A1 (ANXA1) proteins. Solito et al. reported that a treatment with exogenous ANXA1 on human neutrophils stimulated apoptosis [27]. Castro-Caldas et al. reported that dexamethasone and 17β -estradiol promoted ANXA1 secretion in acute T lymphoblastic leukemia cells, CCRF-CEM [28]. Ahmad et al. showed that phytoestrogens induced the production of Annexin A1 in peripheral blood mononuclear cells (PBMC) [29]. However, the underlying mechanism of phytoestrogens that modulate ANXA1 and regulate these processes is still not fully understood. Thus, the knockdown of ANXA1 in this study was to investigate whether the effects of phytoestrogens on apoptosis, cell cycle arrest and phagocytosis are mediated by ANXA1 or occurred independently.

Materials

The chemicals and reagents used in this study were Roswell Park Memorial Institute, RPMI-1640 (Gibco, USA), Iscove's Modified Dulbecco's medium, IMDM (Gibco, USA), L-glutamine (Gibco, USA), fetal bovine serum (Gibco, USA), penicillin-streptomycin antibiotics (Nacalai Tesque, Japan), Silencer® Select Validated siRNA; siRNA ID s1381 (Ambion, USA), INTERFERin® siRNA Transfection reagent (Polyplus Transfection, France), coumestrol (Sigma Aldrich, Germany), daidzein (ChromaDex, USA), genistein (ChromaDex, USA), cyclophosphamide (Baxter Oncology GmbH, Germany), 17β -estradiol (Sigma Aldrich, Germany), phosphate buffered saline, PBS (Amresco, USA), FITC-labeled Annexin V (BD Bioscience, CA), propidium iodide (Sigma Aldrich, Germany), RIPA lysis buffer (Merck, Germany), protease and phosphatase inhibitor (Pierce, USA), Bradford protein assay (Bio-Rad, USA), PVDF membrane (Merck, Germany), tris buffered saline with 0.1% Tween 20, TBST (Bio-Rad, USA), skimmed milk (Nacalai Tesque, Japan), enhanced chemiluminescence (ECL) detection solution (GE Healthcare, Germany), RNase A (Nacalai Tesque, Japan), phorbol 12-myristate 13-acetate, PMA (Tocris, UK), hematoxylin solution (Clin-Tech, UK) and eosin solution (Histo-Line, Malaysia). The primary antibodies used in the western blot assay were caspase-3, caspase-9, caspase-1, Bcl-2, ANXA1, β -actin and the secondary antibody HRP-conjugated anti-rabbit was obtained from Cell Signalling Technology (Danvers, USA).

Methods

Cell culture

The acute myeloid leukemia U937 (ATCC® CRL-1593.2™), acute lymphoblastic leukemia Jurkat (ATCC® TIB-152™), and chronic myeloid leukemia K562 (ATCC® CCL-243™) cells were obtained from American Type Culture Collection (Manassas, VA, USA). The U937 and Jurkat cells were cultured in RPMI 1640 medium and the K562 cells were cultured in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 1% 100 U/mL penicillin and 0.1 mg/ml streptomycin at 37 °C in 5% CO₂, 95% air humidified atmosphere.

Knockdown ANXA1 using small interfering RNA (siRNA)

The Jurkat, U937 and K562 cells (1.5×10^5 cell/ml) were transiently transfected with 40 nM, 80 nM and 100 nM respectively of ANXA1 small interfering RNA (siRNA), (Silencer® Select Validated siRNA; siRNA ID s1381 Ambion, TX, USA) using the INTERFERin transfection reagent (Polyplus-transfection Inc., France) according to the manufacturer's protocol. Both transfection processes were done in serum and antibiotics-free media. Cells were transfected for 72 h before further treatment. After 72 h of ANXA1 transfection, transfected cells were treated with phytoestrogens (coumestrol, daidzein and genistein), 17 β -estradiol and cyclophosphamide (positive control) for 24 h. The concentrations used for treatment with phytoestrogens and 17 β -estradiol, and cyclophosphamide was 40 and 8000 μ g/mL, respectively. These concentrations were selected based on a previous study that showed significant apoptosis in non-transfected leukemic cells (*unpublished data*).

Apoptosis assay

To assess the effect of phytoestrogens on the percentage of apoptosis, the Annexin V/propidium iodide staining was used based on the protocol described by Maioral et al. (2013) with some modifications [30]. After 24 h of treatment, cells were harvested and washed twice with cold PBS. The cell pellets were resuspended in 200 μ L of binding buffer and stained with 10 μ L of propidium iodide and 5 μ L of FITC-labeled Annexin V solution. The sample was incubated for 10 min on ice in the dark according to the manufacturer's protocol. The percentage of viable, apoptotic and necrotic cells was assessed using a flow cytometer (BD FACS Canto II, CA, USA).

Western blotting

The western blotting protocol was based on the general protocol by Bio-Rad, USA with modifications. Cells were lysed with RIPA lysis buffer (MERCK) containing protease and phosphatase inhibitor (PIERCE) for 30 min on ice and then centrifuged at 10000 $\times$ g for 15 min. The total protein content was quantified using Bradford protein assay (Bio-Rad, USA) and denatured in the sample buffer at 95 °C for 5 min. Briefly, 30 μ g of total protein was separated on 10% SDS-PAGE gel at 150 V for 60 min and electrotransferred onto PVDF membrane (MERCK) at 100 V for 75 min. The membranes were blocked with 5% skimmed milk in tris-buffered saline with 0.1% tween 20 (TBST) for 1 h at room temperature and incubated with monoclonal antibodies overnight against caspase-3, caspase-9, caspase-1, Bcl-2, ANXA1 and β β -actin (1:1000) from Cell Signaling Technology (Danvers, USA). Then, the membranes were washed and incubated with secondary antibody HRP-conjugated anti-rabbit (1:5000) obtained from Cell Signalling Technology (Danvers, USA) for an hour. Targeted proteins were visualized using the enhanced chemiluminescence (ECL) detection solution (GE Healthcare, Germany) and band intensities were quantified using ChemiDoc MP System with Image Lab Software (Bio-Rad, USA).

Cell cycle assay

The cell cycle assay was conducted using flow cytometry based on Zbigniew Darynkiewicz's method [31]. After the treatment with phytoestrogens, transfected cells were washed twice with cold PBS and fixed in cold 70% ethanol for 2 h. Subsequently, cells were rinsed with PBS, treated with staining solution containing 0.1% v/v Triton X-100, 0.2 mg/mL of RNase A, and 0.2 mg/mL of PI in PBS for 30 min in the dark room. The distribution of cell cycle phases with different DNA content was determined using flow cytometer (BD FACS Canto II, CA, USA) and analyzed using ModFit LT cell cycle software (ModFit LT, ME USA).

Phagocytosis assay

The phagocytosis assay was adapted from Martinez-Marin's protocol with slight modifications [32]. THP-1 is a human monocytic leukemia cell that can be differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) as a model to study the mechanism of macrophages in removing apoptotic cells [33]. THP-1 cells were treated with 20 ng/ml of PMA and differentiated into macrophages for 48 h. After the treatment with phytoestrogens, transfected cells were co-cultured with macrophages for 3 h. Then, the cells were washed with cold PBS and stained with hematoxylin and eosin. The percentage of phagocytosis and phagocytosis index

were calculated using the following formula: (a) number of macrophages, 200, (b) number of macrophages that engulfed apoptotic cells and (c) number of apoptotic cells engulfed by macrophages as shown below.

$$\text{Percentage of phagocytosis (\%)} = \left(\frac{b}{a}\right) \times 100\%$$

$$\text{Phagocytosis Index (PI)} = \left(\frac{c}{a}\right) \times b$$

Statistical analysis

All data were presented as mean±SEM of three experiments performed in triplicate. The data were analyzed with One-way ANOVA using GraphPad Prism 5 Software (CA, USA) accompanied by Bonferroni's multiple comparison post-test. The optical density of the protein bands detected by Western blotting was normalized based on the β -actin levels. Differences were considered significant if $p < 0.05$ and indicated as follows: ***($p < 0.001$), **($p < 0.01$) and *($p < 0.05$) versus negative control group.

Results

Knockdown of Annexin A1 expression using ANXA1 siRNA

Western blotting was conducted to detect the ANXA1 expression level after the leukemia cells were transiently transfected with ANXA1 siRNA. Figure 1 shows that the expression level of ANXA1 significantly decreased in Jurkat, K562 ($p < 0.05$) and U937 ($p < 0.01$) cells compared to the non-transfected cells. Different concentrations of ANXA1 siRNA with 40 nM, 80 nM and 100 nM were used for Jurkat, U937 and K562 cells, respectively using optimized transfection protocol. The percentage of ANXA1 expression in Jurkat, U937 and K562 was significantly reduced by 70%, 63% and 69% when compared to non-transfected cells, respectively.

Treatment with phytoestrogens reduced ANXA1 expression

The ANXA1-knockdown cells were treated with phytoestrogens (coumestrol, daidzein and genistein) for 24 h and the ANXA1 expression was quantified using Western blot. Figure 2 shows that the ANXA1 level was significantly downregulated in Jurkat cells but no significant effects were observed in K562 cells after treatment with coumestrol, daidzein and genistein ($p < 0.05$). Meanwhile, daidzein caused a significant increase in ANXA1 level in U937 cells ($p < 0.05$) but coumestrol and genistein showed no significant difference.

Effects of phytoestrogens on viability, apoptosis and necrosis in ANXA1-knockdown leukemia cells

The percentage of viable cells after the treatment with genistein significantly decreased (70.0%) with $p < 0.01$ in Jurkat cells compared to the negative control (89.4%). Meanwhile, in U937, estradiol caused significant decrease of percentage of viable cells (55.9%) with $p < 0.001$ compared to its negative control (91.6%). The apoptosis assay was conducted to determine the percentage of apoptosis after the treatment with phytoestrogens on ANXA1-knockdown cells. Following knockdown of ANXA1, phytoestrogens caused an increase in the percentage of apoptosis in Jurkat, K562 and U937 cells. Figure 3 shows that in Jurkat cells, genistein caused significant apoptosis (24.7%) with $p < 0.001$ compared to the negative control (8.1%) while coumestrol, daidzein, 17 β -estradiol and positive control showed no significant effect. K562 cells showed no significant effect in apoptosis after the treatment with coumestrol, daidzein, genistein, estradiol and positive control compared to the negative control. In U937 cells, treatment with 17 β -estradiol (40.8%) caused significant apoptosis with $p < 0.001$ compared to negative control (7.6%) while other compounds had no significant effect. Figure 3 also shows that the treatment with phytoestrogens had no significant difference in necrosis in Jurkat, K562 and U937 cells.

Activation of caspases after treatment with phytoestrogens in ANXA1-knockdown leukemia cells

The extrinsic pathway involves the activation of initiator caspases, caspase-8 and -10 and activates effector caspases, caspase 3 and -7. Meanwhile, apoptosis mediated through intrinsic pathway activates initiator caspase, caspase-9 and activates effector caspases, caspase-3 and -7 [34]. To evaluate the effect of phytoestrogens on ANXA1-knockdown cells, specifically towards the caspase cascade activation in Jurkat, K562 and U937 cells, Western blot was performed. Based on Fig. 4, all tested phytoestrogens increased both procaspase-9 and procaspase-3 expressions in Jurkat and U937 cells. Meanwhile, phytoestrogens did not increase the procaspase-9 level and only increased procaspase-3 level in K562 cells. Yet, no cleavage of caspases was observed after the treatment with phytoestrogens. Daidzein caused an increase in Bcl-2 expression in Jurkat and U937 cells while no changes were observed in K562 cells. Bcl-2 expression in Jurkat and U937 cells was not significantly different after treatment with genistein while the decrease of Bcl-2 expression in K562 cells was not significant. Coumestrol did not increase Bcl-2 expression in all knockdown cells. Coumestrol, daidzein and genistein increased procaspase-1 expression in Jurkat and U937 cells but the increase was not significant. No changes were observed in K562 cells.

Fig. 1 ANXA1 protein expression was downregulated in the transfected cell, T compared to the non-transfected cell, NT (a). The expression level of ANXA1 was quantified using Western blot. β -actin was used as

loading controls. Quantification of ANXA1 protein expression ($*p < 0.05$, $**p < 0.01$) (b). Data are presented as mean $\pm$ SEM with $n = 3$

Fig. 2 Western blotting analysis showed ANXA1 protein expression was regulated after treatment with coumestrol, daidzein and genistein. NC: negative control, PC: positive control, EST: estradiol, COU: coumestrol, DAI: daidzein and GEN: genistein

Fig. 3 The bar chart showed the percentage of viable (a), apoptosis cells (b) and necrosis cells (c) after the treatment with phytoestrogens (coumestrol, daidzein, genistein), positive control (cyclophosphamide) and 17 β -estradiol. Data are presented as mean $\pm$ SEM. * p < 0.05, ** p < 0.01 and *** p < 0.001 versus negative control group. NC: negative

control, PC: positive control, EST: estradiol, COU: coumestrol, DAI: daidzein and GEN: genistein. Fig. S1–S3 show the flow cytometry analysis of cell distribution throughout the quadrant (Q1–Q4) in ANXA1-knockdown Jurkat, K562 and U937 cells

Phytoestrogens caused cell cycle arrest at the G2/M phase in ANXA1-knockdown leukemia cells

To investigate the effect of phytoestrogens in ANXA1-knockdown Jurkat, K562 and U937 cells towards the cell cycle arrest, flow cytometry analysis was conducted. Figure 5 shows that in Jurkat cells, coumestrol caused cell cycle arrests at the G2/M phase (44.2%) with p < 0.001 by a significant decrease in cell population that was in S phase (33.5%) with p < 0.01 compared to the negative control. Estradiol caused cell cycle arrest at G2/M phase (70.2%) with p < 0.001 by a significant decrease in G0/G1 phase cells (7.5%) with p < 0.001; the positive control caused cell cycle arrest at G2/M phase (47.1%) with p < 0.001 by a significant decrease in G0/G1 phase cells (18.1%) with p < 0.01 compared to the negative control. Meanwhile, daidzein and genistein did not cause arrest at any phase in Jurkat cells. In K562

cells, coumestrol caused cell cycle arrest at G2/M phase (55.2%) with p < 0.001 by a significant decrease in G0/G1 phase cells (15.5%) with p < 0.001 and genistein caused cell cycle arrest at G2/M phase (44.7%) with p < 0.01 by a significant decrease in G0/G1 phase cells (3.1%) with p < 0.001 while daidzein produced no significant effect. In U937 cells, only coumestrol caused cell cycle arrest at G2/M phase (82.7%) with p < 0.01 by a significant decrease in G0/G1 phase cells (4.2%) with p < 0.001.

Effect of phytoestrogens on phagocytic activity in ANXA1-knockdown leukemia cells

Based on Fig. 6, treatment with phytoestrogens significantly increased the percentage of phagocytosis in all transfected Jurkat, K562 and U937 cells. Jurkat cells treated with genistein showed the highest percentage of phagocytosis (63.8%),

Fig. 4 The figure shows the expression of caspase-9 (a), caspase-3 (b), Bcl-2 (c) and caspase-1 (d) after the treatment with phytoestrogens (coumestrol, daidzein, genistein), positive control (cyclophosphamide) and 17 β -estradiol from Western blotting and densitometry quantification.

β -actin was used as the endogenous control. Data are presented as mean $\pm$ SEM. * p < 0.05, ** p < 0.01 and *** p < 0.001 versus negative control group. NC: negative control, PC: positive control, EST: estradiol, COU: coumestrol, DAI: daidzein and GEN: genistein

Fig. 5 Cell cycle analysis from flow cytometry histograms showed cells distribution after the treatment with phytoestrogens (coumestrol, daidzein, genistein), positive control (cyclophosphamide) and 17β -estradiol in Jurkat, K562 and U937 cells. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to negative control

and ## $p < 0.01$, ### $p < 0.001$ compared to positive control. NC: negative control, PC: positive control, EST: estradiol, COU: coumestrol, DAI: daidzein and GEN: genistein. Fig. S4–S6 show the flow cytometry analysis of cells distribution throughout cell cycle phases G0/G1, S and G2/M in ANXA1-knockdown Jurkat, K562 and U937 cells

$p < 0.001$) followed by coumestrol (56.8%, $p < 0.01$), daidzein (51.8%, $p < 0.05$) and estradiol (52.3%, $p < 0.05$) compared to the negative control (30.5%). Genistein also caused the highest percentage of phagocytosis in K562 cells (61.7%, $p < 0.001$) followed by coumestrol (53.5%, $p < 0.001$) and daidzein (46.8%, $p < 0.01$) compared to the negative control (31.7%). Estradiol and positive control produced no significant difference compared with the negative control. Meanwhile, U937 cells treated with estradiol had the highest percentage of phagocytosis (51.8%, $p < 0.001$), followed with positive control (40.7%, $p < 0.01$), genistein (35.3%, $p < 0.05$), coumestrol (33.0%, $p < 0.05$) and daidzein (32.3%, $p < 0.05$) compared to the negative control (17.8%).

Phagocytic index (PI) was the highest after the treatment with genistein (54.1, $p < 0.001$), coumestrol (48.3, $p < 0.001$), daidzein (30.5, $p < 0.05$), positive control (28.8, $p < 0.05$) and estradiol (28.1, $p < 0.05$) compared to the negative control (7.2) in K562 cells. Estradiol produced the highest PI in U937 cells (18.6, $p < 0.01$), followed by genistein (8.7, $p < 0.05$), coumestrol (8.4, $p < 0.05$), positive control (7.9, $p < 0.05$) and daidzein (7.7, $p < 0.05$) compared to the negative control (1.8). Genistein yielded the highest PI in Jurkat cells (74.5, $p < 0.001$), daidzein (70.8, $p < 0.001$), coumestrol (35.5, $p < 0.05$) compared to the negative control (8.0).

Discussion

Nowadays, there is an increasing trend for the search of alternative, plant-derived chemotherapeutic drugs with minimum adverse effects and those that target various biological activities in cancer cells. Many reports have shown that phytoestrogens possess anti-cancer properties in various cancer cells [35]. Therefore, we have investigated the effects of phytoestrogens on cell cycle regulation, apoptosis, caspase cascade activation and phagocytosis activities in ANXA1-knockdown leukemic cells.

The ANXA1 protein is an endogenous protein that has implications in apoptosis. High expression of ANXA1 has been found in human leukemic cells [36]. Since these cells display a high level of ANXA1 expression, a knockdown of this protein may be beneficial for leukemia treatment. ANXA1 expression was successfully downregulated in all types of transfected cells. In our laboratory, downregulation of ANXA1 expression induced apoptosis, cell cycle arrest and phagocytosis in leukemic cells (*unpublished data*). Treatment with coumestrol, daidzein and genistein significantly decreased ANXA1 level in transfected Jurkat cells. These findings suggest that coumestrol, daidzein and genistein were capable of further suppressing ANXA1 expression in the

Fig. 6 The bar chart shows the percentage of phagocytosis (a) and phagocytosis index, PI (b) after the treatment with phytoestrogens. Arrows indicate phagocytosis of apoptotic cells at 100X magnification by haematoxylin and eosin (H&E) staining in NC, PC and

phytoestrogens-treated cells (c). NC: negative control, PC: positive control, EST: estradiol, COU: coumestrol, DAI: daidzein and GEN: genistein. Fig. S7–S9 show the phagocytosis activity using H&E staining in ANXA1-knockdown Jurkat, K562 and U937 cells

transfected leukemic cells that already expressed low ANXA1 level, with an exception of daidzein that increased the ANXA1 expression in U937 cells. The decreasing ANXA1 expression by phytoestrogens might influence apoptosis, cell cycle and phagocytosis activities. Therefore, in order to investigate the influence of phytoestrogens on ANXA1 expression during apoptosis activity, a flow cytometric analysis was conducted.

In this study, genistein induced apoptosis in Jurkat cells only. However, coumestrol and daidzein did not cause apoptosis in all ANXA1-knockdown cells. Genistein might exhibit cell type-specific effect as the apoptosis was induced

only in lymphocyte cells and not in myeloid cells (U937 and K562). A previous study reported that high concentrations of genistein induced apoptosis in ER-negative and ER-positive breast cancer cells [37, 38]. In addition, a study done by Balabhadrapathruni (2000) reported that the ineffectiveness of daidzein to inhibit cell proliferation and cell cycle progression or to induce cell death was due to the structure of daidzein that lacks a 5' hydroxyl group on the A ring [19]. These data suggest that ANXA1 plays a vital role in regulating apoptosis in leukemia cells. Western blot was conducted to explain the mechanism of phytoestrogens involved in apoptosis by measuring the caspases, Bcl-2 and ANXA1.

The signaling pathways involved after the treatment with phytoestrogens in apoptosis have been partially elucidated. Since caspases play a major role in apoptosis, we determined whether the treatment with phytoestrogens induces apoptosis via the activation of caspases. It was found that coumestrol, daidzein and genistein did not cleave initiator caspase, caspase-9 and caspase-3, resulting in insignificant percentage of apoptosis. Although the increase was not significant, coumestrol increased caspase-9 and caspase-3 expression in Jurkat and U937 cells, suggesting that this compound might induce apoptosis through intrinsic and extrinsic pathways in both cells. In K562 cells, coumestrol only increased caspase-3 expression and non-significantly induced apoptosis, suggesting that this compound acted through the extrinsic pathway. Daidzein displayed almost the same characteristics with coumestrol: both pathways in U937 and Jurkat cells, and extrinsic pathways in K562 cells. Genistein increased caspase-9 and caspase-3-induced apoptosis through intrinsic and extrinsic pathways in Jurkat cells, increased caspase-9 expression in U937 cells through the intrinsic pathway and K562 cells through the extrinsic pathway.

Bcl-2 family also plays a role in regulating apoptosis. The expression of Bcl-2 after the treatment with coumestrol was not significantly different when compared to the negative control in all types of knockdown cells. Daidzein increased Bcl-2 expression in Jurkat and U937 cells. Meanwhile, genistein decreased Bcl-2 expression in K562 cells. This finding is in agreement with previous studies which reported that genistein also inhibited the growth of MDA-MB-231 cells by inhibiting Bcl-2 expression in Akt or Notch-1 signaling pathway [39, 40]. We found that procaspase-1 expression increased after the treatment with phytoestrogens in Jurkat and U937 cells. In this study, it was demonstrated that the phytoestrogens did not increase procaspase-1 expression in K562 cells. Indeed, no cleavage of caspase-1 was observed after the treatment with phytoestrogens. The activation of caspase-1 will activate not only pro-inflammatory cytokines, pro-IL-1 β and pro-IL-18 but also the effector caspase, caspase-7 [41]. The cleavage of caspase-1 triggers BH3-interacting domain, Bid into truncated Bid, tBid and activates the mitochondrial pathway in cultured mouse cortical neurons [42].

Results obtained showed that coumestrol significantly induced cell cycle arrest at the G2/M phase in all types of transfected cells, suggesting that the accumulation of cell cycle population at G2/M phase was due to DNA damage; the G2/M checkpoints stop the cell cycle process in order to prevent cells from entering mitosis. This finding is supported by Lee et al. (2013) and Lim et al. (2016) who showed that coumestrol inhibited cell growth in breast cancer, colon cancer, cervical and prostate cancer cells [6, 18]. Meanwhile, genistein caused cell cycle arrest at G2/M phase in K562 cells

only and no arrest was observed after the treatment with daidzein. Previous findings showed that genistein had a strong growth inhibitory effect on ATL T cell leukemia cells (Hut102 and ED-40515) compared to non-ATL T cell leukemia cells (Jurkat and Molt-4 cells), indicating that genistein had its specific target molecule in each cell despite the cells being the same type of cancer. Adjakly et al. (2013) showed that daidzein caused cell cycle arrest in various cancer cells whereas Murata et al. (2004) reported that daidzein did not cause DNA damage in rat hepatocytes [43, 44]. Taken together, it is suggested that the accumulation of cell cycle population at G2/M phase was due to DNA damage and that the G2/M checkpoints stopped the cell cycle process in order to prevent cells from entering mitosis. It is evident that coumestrol inhibited cell cycle progression while daidzein did not induce cell cycle arrest in ANXA1-knockdown leukemic cells.

The clearance of apoptotic cells through phagocytosis is a crucial process in maintaining cell homeostasis. The results showed that cells treated with coumestrol, daidzein and genistein facilitated phagocytosis process. Apoptotic cells release signals to attract macrophages to engulf them [45]. The efficiency of a macrophage in removing damaged or apoptotic cells increased after ANXA1 knockdown. The treatment with phytoestrogens caused apoptotic and necrotic cells to exhibit the signals, such as phosphatidylserine (PtdSer) [46]. Besides PtdSer, other signals including sphingosine-1-phosphate (S1P), fractalkine and lysophosphatidylcholine (LPC) have the ability to attract phagocytes to the apoptotic cells [47]. Therefore, signals produced by both apoptotic and necrotic cells attract macrophages to engulf them.

It should be noted that the various effects shown by phytoestrogens and 17 β -estradiol were probably due to the different affinity towards ER α and ER β in different cell types. The expression of ER α and ER β has a distinctive pattern in different cell types, causing various observable effects [48]. Therefore, in all three cell lines studied, the effects of phytoestrogens varied depending on the type of cells.

Conclusion

In summary, genistein was capable to decrease ANXA1 expression and induce apoptosis in ANXA1-knockdown Jurkat cells. However, coumestrol and daidzein were unable to decrease ANXA1, and thus these compounds were incapable to induce apoptosis in ANXA1-knockdown leukemic cells. It can be concluded that the apoptosis and cell cycle arrest produced by phytoestrogens were mediated by ANXA1 protein. Our study provides compelling evidence that these phytoestrogens indirectly induce cell death via suppression of ANXA1, which serves as a potential target for the treatment of leukemia.

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Availability of data and materials All data generated or analyzed during this study are included in this published article and supplementary data.

Authors' contributions MH and EK planned the experimental phase and MH performed the experiments and carried out the statistical analysis. EK, MJ, JAJ and FR conceptualized the study hypothesis and coordinated the research activity. The manuscript was written by MH, EK and reviewed by JAJ, NA and FR. All authors have read and approved the manuscript.

Compliance with ethical standards

Ethical approval Not applicable

Consent for publication Not applicable

Conflict of interest The authors declare that they have no conflict of interest.

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