Induction of cell death and modulation of Annexin A1 by phytoestrogens in human leukemic cell lines

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Abstract

Background: Phytoestrogens are polyphenolic plant compounds which are structurally similar to the endogenous mammalian estrogen, 17β-estradiol. Annexin A1 (ANXA1) is an endogenous protein which inhibits cyclo-oxygenase 2 (COX-2) and phospholipase A2, signal transduction, DNA replication, cell transformation, and mediation of apoptosis.

Objective: This study aimed to determine the effects of selected phytoestrogens on annexin A1 (ANXA1) expression, mode of cell death and cell cycle arrest in different human leukemic cell lines.

Methods: Cells viability were examined by MTT assay and ANXA1 quantification via Enzyme-linked Immunosorbent Assay. Cell cycle and apoptosis were examined by flow cytometer and phagocytosis effect was evaluated using haematoxylin-eosin staining.

Results: Coumestrol significantly (p < 0.05) reduced the total level of ANXA1 in both K562 and U937 cells and genistein significantly (p < 0.05) reduced it in K562, Jurkat and U937 cells, meanwhile estradiol and daidzein induced similar reduction in U937 and Jurkat cells. Coumestrol and daidzein induced apoptosis in K562 and Jurkat cells, while genistein and estradiol induced apoptosis in all tested cells. Coumestrol and estradiol induced cell cycle arrest at G2/M phase in K562 and Jurkat cells with an addition of U937 cells for estradiol. Genistein induced cell cycle arrest at S phase for both K562 and Jurkat cells. However, daidzein induced cell cycle arrest at G0/G1 phase in K562, and G2/M phase of Jurkat cells. Coumestrol, genistein and estradiol induced phagocytosis in all tested cells but daidzein induced significant (p < 0.05) phagocytosis in K562 and Jurkat cells only.

Conclusion: The selected phytoestrogens induced cell cycle arrest, apoptosis and phagocytosis and at the same time they reduced ANXA1 level in the tested cells. The IC50 value of phytoestrogens was undetectable at the concentrations tested, their ability to induce leukemic cells death may be related with their ability to reduce the levels of ANXA1. These findings can be used as a new approach in cancer treatment particularly in leukemia.

Keywords: Annexin A1, Cell cycle arrest, Leukemia, Phagocytosis, Phytoestrogens

1. Introduction

Cell proliferation and cell death are processes that is essential to the development, maintenance of tissue homeostasis and integrity of multicellular organisms. Deregulation of cell cycles and cell death processes such as apoptosis leads to various pathological conditions such as cancer autoimmune diseases, and neurodegenerative diseases (Zaman et al. 2014; Matson & Cook 2017; Jan & Chaudhry 2019).

Leukemia is a group of malignant hematological disease which is mostly due to DNA mutations or chromosomal abnormalities resulting in the loss of control over cell growth by the
hematopoietic stem cells. These abnormalities either activate oncogenic pathways or deactivate tumor suppressor pathways (Liu et al., 2015; Luczak et al., 2012). Generally, leukemia is divided into either acute or chronic before further divided based on the type of cells that undergo malignant transformation (myeloid, myelogenous or lymphocytic). In chronic leukemia, hematopoietic stem cells (HSCs) differentiate and eventually mature but are not fully functional and stay longer in the bloodstream than normal cells. In acute leukemia, HSCs remain as immature state or undifferentiated progenitor cells which resulting in blast cells to reproduce faster (Luczak et al., 2012). Leukemia is the cause of cancer-related deaths in one-third of children and adolescents younger than 15 years (Liu et al., 2015). Dong & Blobe 2006 stated that transforming growth factor-β (TGF-β) were suggested to have an early role in signalling pathway in hematologic malignancies such as leukemia pathogenesis. Resistance towards TGF-β growth-inhibitory and apoptotic effects allows clonal expansion in many hematologic malignancies (Dong and Blobe, 2006). Although hematologic malignancies like leukemia had different pathogenesis and treatment compared to solid tumor as they arise from cells that are readily circulates through the body, they also share many common alterations such as resistance towards growth-inhibitory and differentiation factors, proliferate in the absence of exogenous growth signals and also evade apoptosis and immunosurveillance (Dong and Blobe, 2006). Chemotherapy, radiation, bone marrow transplants and target-based therapies are among current treatment for leukemia (Liu et al., 2015).

Annexin A1 (ANXA1) is an endogenous protein which is one of the members of the Annexin family that bind to calcium and phospholipids which has also been implicated in cell differentiation and proliferation processes, membrane trafficking and cytoskeleton organization (Belvedere et al., 2016; Rong et al., 2014). ANXA1 is either located in cytosol or membrane bound in various types of cell (Lu et al., 2007). Previously known as Lipocortin 1, this 37 kDa protein was originally demonstrated to serve function in down-regulation of inflammation depending on the presence of calcium (Rong et al., 2014; Sheikh and Solito, 2018). Studies showed that ANXA1 is involved in various intra- and extracellular events including inhibition of cyclo-oxygenase 2 (COX-2) and phospholipase A2, signal transduction, DNA replication, cell transformation, and mediation of apoptosis (Sheikh and Solito, 2018; Boudhraa et al., 2016; Ahmad et al., 2014). Recently, it has been suggested that ANXA1 plays a role in cancer cell apoptosis (Lim and Pervaiz, 2007). Its ability to down-regulate COX-2 activity suggests that ANXA1 may be a plausible molecular target for anti-cancer treatment (Hirata, 2014). COX-2 roles in tumorigenesis are upregulating Bcl-2, vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 and –9, epidermal growth factor receptors (EGFR) and extracellular signal-related kinase (ERK). These in return causing apoptosis resistance, induced angiogenesis, and metastases, induced tumor growth and cell invasions. Previous studies showed that inhibition of COX-2 sensitizes cancer cells to treatments (Goradel et al., 2019). Aside from its ability to inhibit COX-2, ANXA1 is reported to regulates multiple proteins that can stimulate or inhibit pathways that contributes to pathogenesis of cancers. Such proteins as TGFβ/SMAD, ERK 1/2, and MMP pathway that are essential in migration, invasion, cell growth and proliferation of cancer cells. ANXA1 is also showed to interact with NFκβ and p21WAF1 that are essential in apoptosis and cell cycle arrest, therefore disrupts apoptosis and cell cycle arrest which leads to cancer progression (Ganesan et al., 2020). Although it is commonly dysregulated in some cancers such as esophageal and prostate cancer, ANXA1 was suggested to be tissue- and cell-type specific protein as it was also reported to be increased in other types of cancer such as colorectal and pancreatic cancer (Rong et al., 2014; Guo et al., 2013). However, there is lack of evidence on its involvement in leukemia. Canaider et al. (2000) demonstrated that endogenous intracellular ANXA1 promoted apoptosis in myelomonocytic-derived cells and macrophages (Canaider et al., 2000). It was also shown that ANXA1 mediates the effect of histone deacetylases (HDAC) inhibitors to inhibit three different types of human leukemic cell lines U937, K562 and Jurkat cells proliferation (Petrella et al., 2008).

Phytoestrogens are polyphenolic non-steroidal plant compounds which are structurally similar to the endogenous mammalian estrogen, 17β-estradiol (Fig. 1). Based on their chemical structure, they can be classified into four main groups namely isoflavones, coumestans, stilbenes and lignans. Although they selectively modulate estrogen receptor activities, each class differently affects the estrogen-mediated pathway (Sirotkin and Harrath, 2014; Hwang and Choi, 2015). Phytoestrogens are commonly present in fruits, vegetables and whole grains that are commonly consumed by human whether as food or medicinal use (Sirotkin and Harrath, 2014). Isoflavones are found in abundance in legumes particularly in soybeans and soy products. Haron et al. (2009 and 2011) stated that raw ‘tempeh’, a popular soy product consumed by the Malaysians, contained 26 ± 6 mg of daidzein and 28 ± 11 mg of genistein in 100 g (wet basis) (Haron et al., 2011; 2009). Konar (2013) demonstrated that coumestrol content was high in red lentils (17.6 ± 1.05 μg/kg) and kidney bean (18.5 ± 1.45 μg/kg) (Konar, 2013). Daidzein is a major isoflavone found in soybean that is metabolized to equol by gut microflora. Equol is considered as the most important metabolite due to a greater affinity for binding to the estrogen receptor than its precursor daidzein. Phytoestrogens such as genistein has been reported to inhibit cancer cell invasion and metastasis (Sirotkin and Harrath, 2014; Hwang and Choi, 2015). Liu et al., 2015 showed that genistein could induce cell-cycle arrest and apoptosis in Jurkat cell lines (Liu et al., 2015).

Previous studies showed that estrogen receptors were also expressed in leukocytes, megakaryocytes and platelets (Du et al., 2017; Pierdominici et al., 2010). Therefore, the present study aimed to investigate the effects of selected phytoestrogens on ANXA1 modulation, apoptosis, cell cycle and phagocytosis in K562, Jurkat and U937 leukemic cell lines.

2. Materials and methods

2.1. Reagents

Coumestrol (Sigma), Daidzein, Genistein, (ChromaDex), Dexamethasone (CCM Duopharma, Malaysia), Estradiol (Sigma), Cyclophosphamide (Endoxan, Halle, Germany), BD Pharmingen™.
Annexin V-FITC (BD Biosciences, CA, USA), Propidium Iodide, PI (Sigma, MO, USA), BD CycleTest™ Plus DNA Reagent Kit (Becton Dickinson, CA, USA), methyl thiazolyltetrazolium (Sigma, MO, USA), Dimethyl sulfoxide, DMSO (Merck, Darmstadt, Germany), ANXA1 ELISA kit (USCNK, Wuhan, China), haematoxylin (CLINETECH LIMITED, GU4 7BN, UK) eosin (HISTO-LINE, Selangor, Malaysia).

2.2. Cell lines

Acute lymphocytic leukemia (Jurkat) and acute myelogenous leukemia (U937) cell lines were purchased from American Type Culture Collection (ATCC, VA, USA) were cultured in RPMI 1640 (Gibco, OK, USA) supplemented with 10% fetal bovine Serum, FBS (Gibco, OK, USA) and 1% Penicillin Streptomycin (Amresco, OH, USA). Chronic myelogenous leukemia (K562), were cultured in Iscove’s Modified Dulbecco’s Medium, IMDM (Gibco, OK, USA) supplemented with 10% fetal bovine Serum (FBS) and 1% Penicillin Streptomycin.

2.3. Cell viability assay

Cell viability was assessed by using protocol from Maioral et al. 2013 with minor modification. Cells were seeded at 2 × 10^5 cells/mL in a 96-well plate and treated with phytoestrogens, estradiol and dexamethasone at concentration ranging from 0 to 320 μg/mL while cyclophosphamide at concentration range of 0–8000 μg/mL and incubated for 24 h at 37 °C. MTT (5 mg/mL) was added to each well in 1:10 ratio. The insoluble formazan was dissolved in dimethylsulfoxide (DMSO) and absorbance was read at 570 nm using a microtiter plate reader. The cell viability was determined using the following formula:

\[
\text{CellViability(\%)} = \frac{A_{\text{treatment}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100
\]

2.4. Quantification of ANXA1 expression

This assay was carried out according to Ahmad et al., 2014 with modification (Ahmad et al., 2014). In final volume of 200 μL, 5 × 10^5 cells/mL were seeded and incubated with 40 μg/mL of coumestrol (149.13 μM), daidzein (157.33 μM), genistein (148.02 μM), estradiol (148.85 μM) and dexamethasone (77.46 μM) at 37 °C for 24 h. The extracellular ANXA1 were collected by centrifuging the cells at 130 × g, 27 °C for 7 min. The cell pellet was re-suspended in 200 μL of PBS with 2 mM EDTA for 5 min at room temperature to collect the membrane-bound ANXA1. Cells were pelleted and the supernatant containing the membrane-bound ANXA1 were collected. 200 μL of RIPA lysis buffer containing protease inhibitor added to the cell pellet and mixture were vortex for 30 s before centrifuged at 13000 × g for 15 min at 4 °C to collect the intracellular ANXA1. The quantification of ANXA1 was performed using ELISA kit for ANXA1 according to the manufacturer’s supplied protocol.

2.5. Apoptosis studies

Cells at concentration of 1 × 10^6 cells per well were treated with 20 and 40 μg/mL of coumestrol (74.57 μM and 149.13 μM), daidzein (78.67 μM and 157.33 μM), genistein (74 μM and 148.02 μM), and estradiol (74.43 μM and 148.85 μM). The treatment concentration was unable to be raised higher due to the insolubility of the phytoestrogens and estradiol in aqueous solution at higher concentration. Cyclophosphamide as a positive control was used at concentration of 8000 μg/mL (28.66 mM) in this assay as at concentration lower than 28.66 mM, no significant growth inhibition was observed. After incubation for 24 h at 37 °C, the cells were washed with cold phosphate-buffered saline (PBS) and re-suspended in Annexin V Binding buffer. Annexin V FITC dye and propidium iodide (PI) were added to the cells according to the manufacturer’s protocol and analyzed with flow cytometer (BD FacsCanto II, CA, USA).

2.6. Analysis of cell cycle

In this assay, 1 × 10^6 cells were treated with coumestrol (149.13 μM), daidzein (157.33 μM), genistein (148.02 μM) and estradiol (148.85 μM), and significant apoptosis of tested cell lines was observed for almost all phytoestrogens against the tested leukemic cell lines. While the positive control, cyclophosphamide was tested at concentration of 28.66 mM as significant apoptosis induction in all cell lines following cyclophosphamide treatment was only observed at this concentration. Cell cycle profiles were evaluated by staining DNA with PI using BD CycleTest™ Plus DNA Reagent Kit according to the manufacturer’s protocol. Samples were analyzed with a flowcytometer (BD FacsCanto II, CA, USA) and ModFit LT (ModFit LT, ME, USA) cell cycle analysis software.

2.7. Phagocytosis assay

The phagocytosis study was carried out by following the method in Fernandez-Boyanapalli et al. 2010 with modification. In this assay, 1 × 10^6 cells were treated with concentration coumestrol (149.13 μM), daidzein (157.33 μM), genistein (148.02 μM), and estradiol (148.85 μM), while the positive control, cyclophosphamide was tested at concentration of 28.66 mM. After 24 h incubation, cells were co-cultured with macrophage for 1 h at 37 °C. Unbound or cells that is not engulfed by the macrophage were washed with ice cold PBS. The cells were fixed with methanol for 20 min prior to washing with tap water. The cells were stained with haematoxylin-eosin method. In each well, 200 macrophages were calculated at random sites under inverted microscope at 1000x magnification. The percentage of phagocytosis = ([macrophage with target cells/200] × 100) and phagocytic index = (percentage of phagocytosis × [average of target cells bound and engulfed/200]) were calculated.

2.8. Statistical analysis

Data were statistically analyzed and presented as mean ± S.E.M. from three independent experiments. Data were analyzed by One Way ANOVA followed by Bonferroni’s post-test using GraphPad Prism 5 software (CA, USA). Differences were considered significant if p < 0.05.

3. Results

3.1. Cell viability assay

The MTT assay was carried out for each compound on selected human leukemic cell lines at the concentration range of 0–320 μg/mL (Fig. 2). Coumestrol showed a dose-dependent effect on each cell type tested, however, in U937 cells, no significant inhibition on cells viability was observed. In K562 and Jurkat cells, coumestrol showed a significant growth inhibition p < 0.001 and p < 0.05 respectively at concentration 20 μg/mL and p < 0.001 starting from concentration 40 μg/mL onwards. Furthermore, daidzein caused significant growth inhibition (p < 0.05) starting from concentration 40 μg/mL onwards only in Jurkat cells. However, genistein only caused significant growth inhibition (p < 0.05) on U937 cells at 320 μg/mL, and on Jurkat cells (p < 0.01) starting from
Moreover, genistein showed more potent effect on K562 cells as significant inhibition (p < 0.001) was observed from concentration 40 μg/mL onwards. Estradiol showed a dose-dependent effect on U937 cells with significant inhibition p < 0.01 was observed starting from 80 μg/mL onward. Significant growth inhibition (p < 0.01) by estradiol in Jurkat cells was observed only at concentration of 320 μg/mL. Cyclophosphamide was tested at different range of concentration due to different cell sensitivity range to the drug. Cyclophosphamide as anticancer drug was unable to induce significant growth inhibition on all three cell lines at concentration lower than 8000 μg/mL (28.66 mM) and only induced significant growth inhibition (p < 0.001) on U937 and Jurkat cells, while p < 0.05 in K562 cells at 8000 μg/mL. Even though significant inhibition was observed in the cell lines treated with the phytoestrogens, the IC50 value of phytoestrogens was unable to be determined at concentration of 320 μg/mL. This is due to the compound property that is insoluble in aqueous solution at high concentration. Therefore, this finding showed that phytoestrogens did not have a cytotoxic effect on leukemic cells at the concentrations tested. Meanwhile the IC50 value of cyclophosphamide was 8213 μg/mL (29.43 mM) for K562 cells, 8844 μg/mL (31.69 mM) for Jurkat cells and 6453 μg/mL (23.12 mM) for U937 cells.

3.2. Quantification of ANXA1 expression

Quantification of ANXA1 protein was performed at treatment with coumestrol (149.13 μM), daidzein (157.33 μM), genistein (148.02 μM), estradiol (148.85 μM) and dexamethasone (77.46 μM). Sheikh & Solito et al. 2018 stated that ANXA1 is a protein that is regulated by glucocorticoid. Thus, Dexamethasone was used in this assay as positive control. In this assay, we quantified extracellular, membrane bound and intracellular ANXA1 after treatment with phytoestrogens, estradiol and dexamethasone and calculated the total concentration of ANXA1 in each leukemic cell lines (Fig. 3).

The concentration of extracellular ANXA1 in K562 cells treated with daidzein (1.26 ± 0.04 ng/mL, p < 0.05) and genistein (1.15 ± 0.06 ng/mL, p < 0.01) showed a significant decrease compared to negative control (2.46 ± 0.07 ng/mL). Likewise, the membrane bound ANXA1 of cells treated by coumestrol 0.69 ± 0.03 ng/mL, genistein 0.76 ± 0.03 ng/mL and estradiol 0.76 ± 0.1 ng/mL showed a significantly lower with p < 0.05 for the three compounds, compared to negative control (1.22 ± 0.04 ng/mL). Intracellular ANXA1 of K562 cells treated with coumestrol (3.39 ± 0.21 ng/mL, p < 0.05), genistein 3.18 ± 0.05 ng/mL and dexamethasone 2.81 ± 0.06 ng/mL with p < 0.01 for both compounds, were significantly lower compared to negative control (4.47 ± 0.11 ng/mL). Total ANXA1 in the K562 cells treated by coumestrol (5.60 ± 0.19 ng/mL) and genistein (5.10 ± 0.03 ng/mL) was significantly lower with p < 0.001 for both compounds, compared to the negative control (8.15 ± 0.04 ng/mL). A significant reduction in total ANXA1 (p < 0.05) was also observed in dexamethasone (6.84 ± 0.11 ng/mL) treated K562 cells compared to negative control. The extracellular ANXA1 in Jurkat cells treated by daidzein (0.45 ± 0.00 ng/mL) and genistein (0.48 ± 0.03 ng/mL) was significantly lower with p < 0.05 for both compounds compared to negative control (0.94 ± 0.15 ng/mL). However, there was no significant modulation on membrane bound ANXA1 in Jurkat cells treated with phytoestrogens, estradiol and dexamethasone. Only coumestrol showed a significant reduction (p < 0.05) of the intracellular ANXA1 in Jurkat cells (1.69 ± 0.11 ng/mL compared to the negative control (2.84 ± 0.31 ng/mL). The total ANXA1 was significantly (p < 0.01, for both compounds) reduced in Jurkat cells treated by daidzein (2.86 ± 0.06 ng/mL), genistein (2.90 ± 0.08 ng/mL) and dexamethasone, 3.42 ± 0.07 ng/mL (p < 0.05) compared to negative control (4.37 ± 0.25 ng/mL).
In U937 cells treated with phytoestrogens, estradiol and dexamethasone, the extracellular and membrane bound ANXA1 levels were not significantly modulated. Contrary, a significant decrease of intracellular ANXA1 concentration was observed in U937 cells treated with coumestrol (1.74 ± 0.11 ng/mL) and genistein (1.75 ± 0.10 ng/mL) with p < 0.05 for both compounds, and with p < 0.001 for dexamethasone (1.37 ± 0.07 ng/mL) compared to negative control (2.41 ± 0.16 ng/mL). In comparison to positive control in U937 cells, dexamethasone decreased intracellular ANXA1 level stronger than daidzein (2.74 ± 0.03 ng/mL) with p < 0.001 and p < 0.01 for estradiol (2.08 ± 0.14 ng/mL) was observed. The total ANXA1 was significantly lower compared to the negative control (3.34 ± 0.11 ng/mL) in U937 cells treated by coumestrol, 2.70 ± 0.14 ng/mL (p < 0.01), genistein, 2.57 ± 0.03 ng/mL (p < 0.001), estradiol, 2.88 ± 0.07 ng/mL (p < 0.05) and dexamethasone, 2.17 ± 0.03 ng/mL (p < 0.001). This finding demonstrated that genistein decreased significantly the total ANXA1 concentration in all cell lines tested, meanwhile, coumestrol decreased in K562 and U937 cell lines and daidzein decreased only in Jurkat cell.

3.3. Effect of phytoestrogens on apoptosis induction in K562, Jurkat and U937 cell lines

This assay was carried out using flowcytometric analysis by dual staining of the cell lines with Annexin V-FITC and PI to determine the percentage of apoptotic and necrotic cells. The cell lines were treated with two concentrations of coumestrol (74.57 µM and 149.13 µM), genistein (74 µM and 148.02 µM), daidzein (78.67 µM and 157.33 µM), and estradiol (74.43 µM and 148.85 µM) (Fig. 4A and 4B). For positive control cyclophosphamide, the tested concentration was 28.66 mM as this drug showed no growth inhibition at concentration lower than 28.66 mM.

Concentration 74.57 µM of coumestrol induced apoptosis in the Jurkat cells (10.27 ± 0.50%) significantly with p < 0.001, meanwhile at concentration 149.13 µM, it induced apoptosis in both K562 cell (21.47 ± 1.14%, p < 0.01) and Jurkat cells (12.73 ± 0.29%, p < 0.001) compared to its negative control. The same time coumestrol caused slightly necrosis to the Jurkat cells (9.23 ± 2.43%) with
p < 0.05. The daidzein at concentration 78.67 μM induced apoptosis at the Jurkat cells (10.30 ± 0.62%, p < 0.001) and at concentration of 157.33 μM, it induced apoptosis significantly in the K562 cells (21.37 ± 2.45%, p < 0.01) and Jurkat cells (9.30 ± 0.36%, p < 0.05), meanwhile it caused necrosis at the Jurkat cells (9.67 ± 0.34%, p < 0.01) compared to negative control. The genistein induced apoptosis significantly at all cell lines tested as the K562 cells (25.80 ± 2.28%, p < 0.01), the Jurkat cells (9.63 ± 0.35%, p < 0.01) and U937 cells (17.27 ± 1.31%, p < 0.001) at concentration of 74 μM and at concentration of 148.02 μM, genistein also induced apoptosis significantly at all cell lines tested, for the K562 cell (29.43 ± 3.70%), the Jurkat cells (14.40 ± 1.83%) and for the U937 cells (27.37 ± 3.62%) with p < 0.001 for the three cell lines compared to their negative controls. More than that genistein did not cause necrosis to all cell lines tested. Estradiol as endogenous hormone induced apoptosis significantly with p < 0.001 in all cell lines tested except for the K562 and Jurkat cells with p < 0.01 at concentration of 148.85 μM. This hormone also caused necrosis significantly in the K562 cells (6.80 ± 0.80%) with p < 0.05 at concentration 148.85 μM. The positive control cyclophosphamide induces apoptosis significantly higher for all cell lines tested (p < 0.01 to p < 0.001, 44.70 ± 1.11%) compared to the negative control, coumestrol, daidzein, genistein and estradiol, meanwhile it also caused necrosis in the U937 cell line even though it was insignificant.

Our finding showed a variation of phytoestrogens ability in inducing apoptosis, where genistein is strongest and followed by coumestrol then daidzein, more than that genistein did not cause necrosis in all cell lines tested. The cell lines response to the phytoestrogens also varied as all the cell lines tested were sensitive to genistein while the Jurkat cells was sensitive to coumestrol. The K562 cells showed higher sensitivity to genistein and the same sensitivity to coumestrol and daidzein. However, the U937 showed high sensitivity against genistein but resistant to coumestrol and daidzein. K562 cells showed higher sensitivity to genistein and same sensitivity to coumestrol and daidzein.

3.4. Cell cycle arrest induction in K562, Jurkat and U937 cell lines.

Cell cycle is a mechanism that is responsible for duplicating eukaryotic cells, when irreparable damage occur in the DNA, will cause arrest in this process that leads to apoptosis initiation. To determine the effects of phytoestrogens and estradiol on the tested cell lines, flowcytometric analysis based on propidium iodide stained nucleic cells was performed. In the current study, we determined the effects coumestrol (149.13 μM), daidzein (157.33 μM), genistein (148.02 μM), and estradiol (148.85 μM) treatment on cell cycle arrest on the K562, Jurkat and U937 cell lines (Fig. 5).

Coumestrol caused significant cycle arrest (p < 0.001) at G2/M phase on the K562 (34.58 ± 2.25%) and Jurkat (41.73 ± 2.76%) cells, and significant reduction (p < 0.01) on S phase (39.85 ± 3.95%) and (39.67 ± 2.10%, p < 0.01) respectively compared to their negative control (G2/M: 15.44 ± 0.17%; S: 53.07 ± 0.75%). The U937 cells showed that coumestrol significantly increased (p < 0.001) the percentage of cells in both S 72.21 ± 1.64% and G2/M 25.41 ± 2.94% phases while reducing cells in the G0/G1 phase 2.39 ± 1.32% (p < 0.001) compared to their negative control (G0/G1: 42.49 ± 0.64%; S: 56.77 ± 1.28%; G2/M: 14.94 ± 0.59%). Interestingly daidzein...
induced significant cycle arrest at G0/G1 phase (53.06 ± 2.18%, p < 0.001) in the K562 cells with significant reduction in S phase (31.68 ± 0.23%, p < 0.001) compared to the negative controls. Treatment of K562 and Jurkat cells with genistein showed the cycle arrest at S phase, 68.82 ± 3.08% (p < 0.05) and (74.39 ± 2.35%, p < 0.01) respectively and accompanied by reduction at G0/G1 phase, 18.31 ± 1.63% (p < 0.05) and (14.22 ± 3.09%, p < 0.01) respectively compared to their negative control. Meanwhile genistein showed no significant effect on the cell cycle of the U937 cells.

Estradiol caused cycle arrest on K562 and U937 cells at G2/M phase (37.24 ± 2.71%, p < 0.001) and (49.99 ± 1.12%, p < 0.001) respectively and reduce G0/G1 phase (15.03 ± 2.74%, p < 0.01) and (14.29 ± 1.84%, p < 0.001) respectively compared to their negative control. In Jurkat cells, estradiol induced significant cycle arrest at G2/M phase (47.98 ± 4.14%, p < 0.001) and compensated by significant reduction at S phase (42.52 ± 2.45%, p < 0.05) and at G0/G1 phase (15.03 ± 2.74%, p < 0.01) and (14.29 ± 1.84%, p < 0.001) respectively compared to their negative control. In Jurkat cells, treatment with coumestrol showed the percentage of phagocytosis significantly increased of 39.83 ± 0.73% (p < 0.01), and daidzein (54.83 ± 3.88%), genistein (44.67 ± 1.17%) and estradiol 48.17 ± 2.13% (p < 0.001 for the three compounds) compared to the negative control (26.50 ± 0.76%), with only daidzein showing significantly higher in percentage (p < 0.01) compared to the positive control (39.00 ± 0.76%). In U937 cells, the percentage of phagocytosis caused by coumestrol (38.17 ± 0.88%), genistein (32.83 ± 1.48%) and estradiol (52.00 ± 1.53%) showed significant higher with p < 0.001 for the three compounds compared to negative control.

3.5. Phagocytosis analysis

Phagocytosis is the final process of programmed cell death to prevent occurrence of secondary necrosis that can leads to inflammation and autoimmunity (Poon et al., 2014; Arandjelovic and Ravichandran, 2015). In this study, cells were stained with haematoxylin-eosin to differentiate the apoptotic cells, macrophages and phagocytosed cells and percentage of phagocytosis along with phagocytic index was calculated (Fig. 6).
Estradiol showed comparable phagocytosis induction with the positive control (56.00 ± 1.32%).

In the K562 cells, phagocytic index (PI) of coumestrol (32.34 ± 2.81, p < 0.01), estradiol (29.41 ± 7.86 (p < 0.05) and daidzein (41.68 ± 4.12, p < 0.001) showed significant increase compared to the negative control (6.34 ± 0.77), moreover it was a comparable effect to the positive control (35.13 ± 1.82). In Jurkat cells, coumestrol (28.86 ± 1.77) and, genistein (31.57 ± 3.62) with p < 0.01 for both compounds and estradiol (38.02 ± 3.22, p < 0.001) induced significantly higher PI compared to the negative control (7.00 ± 0.60) with no significant difference observed when compared with the positive control (31.42 ± 2.39). Meanwhile, increase in the PI induced by daidzein, 69.69 ± 4.97 (p < 0.001) was significantly higher compared to both negative and positive controls. Significantly higher PI was observed in U937 cells treated with coumestrol (25.34 ± 1.02, p < 0.01), genistein (38.82 ± 2.51) and estradiol 45.48 ± 1.83 with p < 0.001 for both compounds compared to the negative control (9.79 ± 0.49) while significantly lower (p < 0.001) compared to the positive control (70.25 ± 3.86). This finding showed that all the compounds tested increased the phagocytosis process in the three cell lines except for daidzein in the U937 cells.

3.6. Relationship of ANXA1 modulation with cell death induced by phytoestrogens

3.6.1. Coumestrol

In K562 cells treated with coumestrol at 149.13 μM, significant reduction in membrane bound, intracellular and total ANXA1 were observed at the same time significant increase in apoptosis was also observed along with significant increase in percent of phagocytosis and phagocytosis index. Cell cycle arrest at G2/M phase was also observed. These results suggested that may reduction of membrane bound, intracellular and total ANXA1 in K562 cells treated with coumestrol led to cell cycle arrest at G2/M phase, thus significantly increased apoptosis along with phagocytosis of this cells. While in Jurkat cells, coumestrol reduced intracellular ANXA1 significantly while causing significant increase of apoptosis, phagocytosis and inducing cell cycle arrest at G2/M phase. Taken together, these results showed that coumestrol exerts its effect in inhibiting significant...
Jurkat cells by reducing intracellular ANXA1 which led to cell cycle arrest and thus triggering apoptosis and phagocytosis of this cells. Coumestrol significantly reduced intracellular and total ANXA1 in U937 cells. However, this did not trigger cell cycle arrest which led to no significant apoptosis. In U937 cells, although coumestrol caused no significant apoptosis, significant phagocytosis was observed. Collectively, these results suggest that coumestrol effects on ANXA1 of U937 cells was not related to U937 cells' cell cycle observed. Collectively, these results suggest that coumestrol effects on ANXA1 of U937 cells was not related to U937 cells' cell cycle arrest.

3.6.2. Daidzein

Daidzein at 157.33 μM significantly reduced extracellular ANXA1 of K562 cells. Significant increase of apoptosis and phagocytosis with cell cycle arrest at G0/G1 were also observed. This suggest that cell cycle arrest by daidzein caused significant apoptosis in K562 cells which led to increase in phagocytosis of this cells. This effect might be related to the reduction of extracellular ANXA1 in these cells by daidzein treatment. In Jurkat cells, daidzein caused significant reduction of both extracellular and total ANXA1. Based in the observed results, these reductions led to cell cycle arrest of Jurkat cells at G2/M phase. This then triggered significant apoptosis and thus significant increase in Jurkat cell phagocytosis. Unlike in K562 and Jurkat cells, daidzein did not exerts any significant effects on U937 cells.

3.6.3. Genistein

K562 cells treated with 148.02 μM of genistein showed significant reduction in extracellular, membrane bound, intracellular and total ANXA1. Cell cycle arrest at S phase, followed by significant increase in apoptosis and thus significant phagocytosis was also observed. Taken together, these results suggested that genistein inhibited K562 cells by significantly reduced ANXA1 which triggered cell cycle arrest and led to apoptosis and phagocytosis. Genistein significantly reduced extracellular and total ANXA1 of Jurkat cells and triggered cell cycle arrest at S phase then led to apoptosis and thus caused significant increase in phagocytosis. While in U937 cells, intracellular and total ANXA1 was significantly reduced by genistein and it also induced significant apoptosis and phagocytosis on U937 cells treated with genistein was also observed. These results suggested that significant reduction of ANXA1 by genistein in U937 cells directly triggered apoptosis of this cells without causing cell cycle arrest.

4. Discussion

Apoptosis is a programmed cell death that is a mechanism which removed the excessively damaged or potentially harmful cells which is vital in maintaining cellular and tissues homeostasis, fully functional capacity of the immune system and normal organs (Fraser et al., 2009; Skommer et al., 2010). Apoptosis can be triggered via two pathways: the extrinsic pathway which involved activation of the death receptors and the intrinsic pathway which is also known as the mitochondrial-mediated pathway (Tang et al., 2013; Jassamai et al., 2016). This study demonstrated that coumestrol and daidzein induced apoptosis in the K562 and Jurkat cells, while genistein induced apoptosis in K562, Jurkat and U937. This is in line with previous studies that stated phytoestrogens could inhibit various cancer cells (Sirokint and Harrath, 2014; Hwang and Choi, 2015). Many studies reported that these three phytoestrogens mediated its apoptosis effect via the mitochondrial-mediated (intrinsic) pathway (Zafar et al., 2017; Jin et al., 2010). Coumestrol (Zafar et al., 2017; Jin et al., 2010), daidzein (Jin et al., 2010) and genistein (Li et al., 2010) were shown to up-regulates the production of reactive oxygen species (ROS). Increased in ROS leads to the loss of the mitochondrial membrane permeability which caused released of cytochrome c and induced apoptosis (Zafar et al., 2017; Lim et al., 2017). Coumestrol also may induce apoptosis by inhibiting casein kinase II (CKII), which play roles in cell viability and survival, apoptosis suppression, RNA synthesis and cell transformation (Lee et al., 2013; Liu et al., 2013). Liu et al 2013 showed that coumestrol inhibition of CKII that leads to inhibition of Akt/Pkb pathways that is involved in anti-apoptotic pathways and suppression of caspase activity (Liu et al., 2013). Bcl-2 is an anti-apoptotic protein that is highly expressed in cancer cells and maintains the integrity of the mitochondrial. Coumestrol, daidzein and genistein previously have been shown to down-regulate Bcl-2 expression in human hepatoma, hepatic and breast cancer cells (Jin et al., 2010; Li et al., 2012; Park et al., 2013). Down-regulation of Bcl-2 led to loss of mitochondrial integrity and released of cytochrome c into the cytosol. This initiated the caspase cascade apoptosis induction (Skommer et al., 2010). Previous studies also showed that treatment of human hepatoma, prostate and hepatic cancer cells with coumestrol, daidzein and genistein up-regulate the caspase-3, –9 and –7 which marks the intrinsic apoptosis pathways (Lee et al., 2013; Li et al., 2010; Park et al., 2013). Like genistein, estradiol also caused significant apoptotic effect of K562, Jurkat and U937 cells. Previous study stated that estradiol at pharmacological dose inhibited proliferation of murine leukemia cells. Sex steroid such as estrogens and progest-
Shapiro and Harper, 1999). Estrogens and flavonoids that ligate to type-2 EBS were able inhibit CDK1 which mediated the G2/M progression (Mossuz et al., 1998). p53 also played roles in G2/M arrest by inducing both 14–3-3ζ and p21Waf1/Cip1 that maintained the G2/M arrest by preventing the activation of cdc2 (Pieterpen and Stewart, 2002; Shapiro and Harper, 1999). In the current study, we also demonstrated that daidzein induced cell cycle arrest in K562 cells at G0/G1 phase. This result is concurrent with previous studies that showed daidzein caused cell cycle arrest at the G0/G1 phase in human melanoma cells (Casagrande and Darbon, 2001), choriocarcinoma cells (Zheng et al., 2017) and bladder cancer cells (He et al., 2016). Daidzein also induced G0/G1 arrest in the colon cancer, cervical cancer, hepatic and gastric carcinoma (Zheng et al., 2017). Daidzein treatment increased the expression of p21, a cyclin dependent kinase inhibitor which were correlated with inhibition of CDK 4 and CDK2 which are essential in the progression of G0/G1 phase. Daidzein also down-regulated the cyclin D1, c-myc and proliferation nuclear antigen (PCNA) which plays roles in the progression of G0/G1 phase (Zheng et al., 2017; Casagrande and Darbon, 2001). In the current study, unlike in K562 cells, daidzein induced apoptosis in Jurkat cells while causing cell cycle arrest at G2/M phase. This was in line with finding of Han et al. (2015) showed daizain causes G2/M arrest on hepatocellular BEL-7402 cell lines (Han et al., 2015). Study by Guo et al. (2004) also showed that, daidzein could cause cell cycle arrest at either G0/G1 or G2/M phase (Guo et al., 2004). We also demonstrated that genistein induced cell cycle arrest at S phase in K562 and Jurkat cells. Traganos et al. (1992) stated that genistein inhibition of tyrosine phosphorylation during the late stage of S phase may slow or block the transition of the cells from S phase to G2/M phase (Traganos et al., 1992). Previous study by Li et al. (2010) demonstrated that genistein at low concentration (5–10 μg/mL) induced cell cycle arrest at G2/M phase, while increasing the dose to much higher concentration led to perturbation of the cell cycle at the S phase (Li et al., 2010). S phase progression is regulated by cyclin A, CDK2 and E2F-DP heterodimeric complex. Failure to inactivate the E2F-DP-1 will result in persistency activity of the E2F-1 which leads to delay or block in the S phase. Inhibition of cyclin A-CDK2 complexes also leads to S phase arrest. Cell cycle arrest in S phase leads to induction of apoptosis (Pucci et al., 2000; Shapiro and Harper, 1999).

Dead cells are rapidly removed in order to eliminate possibility of development of secondary necrosis and leakage of cellular content via phagocytosis (Poon et al., 2014; Arandjelovic and Ravichandran, 2015). In the current study, coumestrol, genistein and estradiol induced significant phagocytosis of K562, Jurkat and U937 cells, while daidzein in K562 and Jurkat cells only. Except for coumestrol in U937 cells, these results are in line with the results of the apoptosis induction assay by these compounds. Dead cells produce various ‘find me’ and ‘eat me’ signal that attract the phagocytes (Poon et al., 2014; Arandjelovic and Ravichandran, 2015). Phosphatidylserine (PtdSer) is the most common ‘eat me’ signal by the apoptotic and necrotic cells (Poon et al., 2014; Arandjelovic and Ravichandran, 2015). PtdSer is externalised on the membrane of the cells and trigger the phagocytic uptake (Poon et al., 2014). Apart from PtdSer, complement component C1q, collectin mannose binding lectin (MBL), released of fractalkine, lysophosphatidylcholine (LPC) and sialic acid-1–phosphate (S1P) also stimulate and direct the monocytes and macrophages to the target cells (Poon et al., 2014; Arandjelovic and Ravichandran, 2015).

ANXA1 concentration in the leukaemic cell as K562, Jurkat and U937 cells were significantly higher compared to the ANXA1 level in peripheral blood mononuclear cells (2.57 ± 0.13 ng/mL) (Sabran et al., 2019). That study was in line with the current results that found the ANXA1 concentrations in the negative control were higher than PBMC and demonstrated that treatment of these cell lines with phytoestrogens and estradiol significantly reduced the ANXA1 concentrations. The reduction of the ANXA1 in genistein and estradiol treated K562, Jurkat and U937 cells was accompanied by increased in cell cycle arrest, apoptosis and phagocytosis of these cells. This can also be observed in daidzein treated Jurkat cells and coumestrol treated K562 cells. High expression of ANXA1 in RAW macrophages leads to constitutive activation of the ERK, which is responsible for cells proliferation and survival (Iseki et al., 2009). Up-regulation of ANXA1 inhibited apoptosis in human leukemic cells induced by the tumor necrosis factor and protects leukemic blast from immune mediated killing (Wu et al., 2000). In the current study, the tested cell lines treated with dexamethasone showed significant reduction in the total ANXA1 levels. This was in concurrent with study by D’Acquisto et al. (2000) that showed dexamethasone inhibit ANXA1 protein and mRNA expression in CD4 + cells in a time-dependent manner (D’Acquisto et al., 2008). Kamal et al. (2001) also stated that ANXA1 induction by glucocorticoid could be depending on specific cell type and systems (Kamal et al., 2001). Therefore, there is possibility that the reduction in ANXA1 in these cells by the phytoestrogens and estradiol enhanced the cell cycle arrest and apoptosis induction of the cells and thus increased the percentage of the phagocytosis. Considering the IC50 value of phytoestrogens are undetectable at the concentrations tested, their ability to induce leukemic cells death may be related with their ability to reduce the levels of ANXA1.

5. Conclusions

The selected phytoestrogens induced cell cycle arrest, apoptosis and phagocytosis and at the same time reduced ANXA1 level in the tested cells. The leukemic cells showed the variation in sensitivity to different phytoestrogens. Since the phytoestrogens did not demonstrate cytotoxic effect which was shown by their IC50 values were undetectable at the concentrations tested, their ability to induce leukemic cells death may be related with their ability to reduce the levels of ANXA1. This finding can be used as a new approach in cancer treatment particularly in leukemia.

6. Data availability

All data generated or analyzed during this study are included in this published article and supplement data (Fig. S1).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors’ Contributions
AS performed the experiments and carried out statistical analysis. AS and EK planned the experimental phases and analyzed the data. EK, NA and MJ conceptualized the study hypothesis and coordinated the research activity. The manuscript was written by AS, EK and reviewed by JA1 and IBJ. All authors have read and approved the manuscript.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2020.12.011.

References
Ahmad, W., Kumolosasi, E., Jantan, I., Jasamai, M., Salim, E., 2014. Modulatory effect of phytoestrogens and curcumin on induction of Annexin 1 in human peripheral blood mononuclear cells and their inhibitory effect on secretory phospholipase A2. Trop. J. Pharm. Res. 13, 217–177.

Arandjelovic, S., Ravichandran, K.S., 2015. Phagocytosis of apoptotic cells in homeostasis. Nature Immumol. 16, 907–917.

Azmi, N., Chee, S.H., Mohd Faizi, N., Jasamai, M., Kumolosasi, E., 2018. Viability and apoptosis effects of green tea (Camellia sinensis) methanol extract on human leukemic cell line. Acta Pharm. Biol. 75 (1), 51–58.

Belvedere, R., Bizzarro, V., Forte, G., Dal Piaz, F., Parente, L., Petrella, A., 2016. Annexin A1 contributes to pancreatic cancer cell phenotype, behaviour and metastatic potential independent of Fornyl Peptide Receptor pathway. Sci. Rep. 6, 1–14.

Blagosklonny, M.V., Neckers, L.M., 1994. Cytostatic and cytotoxic activity of sex steroids against human leukemia cell lines. Cancer Lett. 76 (2–3), 81–86.

Boudhraa, Z., Bouchon, B., Viaillard, C., D’Incan, M., Degoul, F., 2016. Annexin A1 localization and its relevance to cancer. Clin. Sci. 130, 205–220.

Canaider, S., Solito, S., De Coupade, C., Flower, R.J., Russo-Marie, F., Goulding, N.J., 2005. Annexin A1 and its functions: clinical potential. Nat. Rev. Urol. 2, 343–350.

Han, J.B., Li, W., Jiang, G.B., Lai, S.H., Zhang, C., 2005. Anticancerogenic effect of dietary phytoestrogens on human leukemic mononuclear cells. Nutr. Cancer 67 (5), 796–803.

Iseki, Y., Imoto, A., Okazaki, T., Harigae, H., Takahashi, S., 2009. Identification of annexin 1 as a PU.1 target gene in leukemia cells. Leuk. Res. 33 (12), 1658–1663.

Jian, R., Chaudhary, G.E., 2019. Understanding apoptosis and apoptotic pathways targeted cancer therapeutics. Adv. Pharm. Bull. 9 (2), 205–218.

Jin, S., Zhang, Q.Y., Kang, X.M., Wang, J.X., Zhao, W.H., 2010. Daidzein induces MCF-7 breast cancer cell apoptosis via the mitochondrial pathway. Ann. Oncol. 21 (2), 263–268.

Kamal, A.M., Smith, S.W., Wijayasinghe, M.D.S., Solito, E., Corrigan, C.J., 2001. An annexin I (ANX1)-derived peptide inhibits prostate antigen-driven human T cell Th1 and Th2 responses in vitro. Clin. Exp. Allergy 31 (7), 1116–1125.

Konar, N., 2013. Non-isolavone phytoestrogenic compound contents of various legumes. Eur. Food Res. Technol. 236, 523–530.

Lee, Y.H., Yuk, H.J., Park, K.H., Bae, Y.S., 2013. Coumestrol induces senescence through protein kinase CKII inhibition-mediated reactive oxygen species production in human breast cancer and colon cancer cells. Food Chem. 141 (4), 1351–1356.

Li, W., Frame, L.T., Hirsch, S., Cobos, E., 2010. Genistein and hematological malignancies. Cancer Lett. 296 (1), 1–8.

Li, H.Q., Luo, Y., Qiao, C.H., 2012. The mechanisms of anticancer agents by genistein and its synthetic derivatives. Molecules. 17 (4), 350–362.

Lim, W., Jeong, M., Bazer, F.W., Song, G., 2017. Coumestrol induces proliferation and migration of prostate cancer cells by regulating AKT, ERK1/2, and JNK MAPK cell signaling cascades. J. Cell. Physiol. 232, 862–871.

Liu, L.H.K., Pervaiz, S., 2007. Annexin 1: the new face of an old molecule. FASEB J. 21, 968–975.

Liu, X., Ye, F., Wu, J., How, B., Li, Wang, Z.Y., 2015. Signaling proteins and pathways affected by flavonoids in leukemia cells. Nutr. Cancer. 67, 238–249.

Lu, S., He, J., Yang, Y.L., Xu, Z., Petro, C., Jablons, D.M., You, L., 2013. Coumestrol from the national cancer Institute’s natural product library is a novel inhibitor of protein kinase CK2. BMC. Pharmacol. Toxicol. 14 (36).

Liu, Q.Y., Jin, Y.S., Zhang, Z.F., Le A.D., Heber, D., Li, F.P., Dubernet, M., Rao, J.Y., 2007. Green tea induces annexin-1 expression in human adenocarcinoma A549 cells: involvement of annexin-1 in actin remodelling. Lab. Invest. 87, 456–465.

Luzak, M., Kazmierczak, M., Handshich, L., Lewandowski, K., Komornicki, M., Figiwerowicz, M., 2012. Comparative proteome analysis of acute myeloid leukemia with and without maturation. J. Proteomics 75, 5734–5748.

Maioral, M.F., Gaspar, P.C., Souza, G.R.R., Mascarello, A., Chiaradia, L.D., Licinio, M.A., Moraes, A.C.R., Nunes, R.A., Nunes, R., Santos-Silva, M.C., 2013. Apoptotic events induced by synthetic naphthylchalcones in human acute leukemia cell lines. Biochemistry 95 (4), 866–874.

Matson, J.P., Cook, J.G., 2017. Cell cycle proliferation decisions: the impact of single cell analyses. The FEBs journal. 284 (3), 362–375.

Moscovici, E., Cousin, F., Camacho, M.F., Polack, B., Sotto, J.J., Kolodie, L., 1998. Effects of two sex steroids (17β estradiol and testosterone) on proliferation and clonal growth of the human mononuclear leukemia cell line, U937. Leuk. Res. 22 (11), 1063–1072.

Park, H.J., Jeon, Y.K., You, J.C., Kim, K.J., 2013. Daidzein causes cytochrome c-mediated apoptosis via the Bcl-2 family in human hematopoietic cancer cells. Food Chem. Toxicol. 60, 542–549.

Petrella, A., D’Acunto, C.W., Rodriguez, M., Ratta, M., Tosco, A., Bruno, I., Tarennaro, S., Taddei, M., Paloma, L.G., Parente, L., 2008. Effects of FR235222, a novel HDAC inhibitor, in proliferation and apoptosis of human leukaemia cell lines. Role of Annexin. Eur. J. Cancer 44 (5), 740–749.

Pierdominici, M., Maselli, A., Colananti, T., Giannamore, A.M., Delunardo, F., Vacirca, D., Sanchez, M., Giovanniotti, A., Malorni, W., Ortona, E., 2010. Estrogen receptor profiles in human peripheral blood lymphocytes. Immunol. Lett. 132 (1–2), 79–85.

Petenpol, J.A., Stewart, Z.A., 2002. Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. Toxicology 181–182, 475–481.

Poon, I.K.H., Lucas, C.D., Rossi, A.G., Ravichandran, K.S., 2014. Apoptotic cell clearance: basic biology and therapeutic potential. Nat. Rev. Immunol. 14, 166–180.

Rong, B., Cai, X., Yang, S., 2014. Annexin A1 in malignant tumors: current opinions and controversies. Int. J. Biol. Markers 29, e8–e20.

Sabran, A., Kumolosasi, E., Jantan, I., 2019. Effects of Annexin A1 on apoptosis and proliferation of human leukemic cell lines. Acta Pharm. 70, 75–86.

Shapiro, G.I., Harper, J.W., 1999. Anticancer drug targets: cell cycle and checkpoint pathways. Int. J. Cancer 83, 83–88.

Sheikh, M.H., Solito, E., 2018. Annexin A1: Uncovering the many talents of an old player. Targeted cancer therapeutics. Adv. Pharm. Bull 9 (2), 205–218.

Smokmer, J., Brittain, T., Raychaudhuri, S., 2010. Bcl-2 domain induced apoptosis by increasing the time-to-death and intrinsic cell-to-cell variations. Apoptosis 15 (10), 1223–1233.

Ye, H., Wu, X., Cao, L., Hou, Y., Chen, H., Wu, L., Liu, Z., Zhu, W., Gu, Y., 2016. Daidzein exerts anti-tumor activity against bladder cancer cells via inhibition of FGF3 receptor. Neoplasma. 63 (4), 523–531.
Tang, S., Hu, J., Meng, Q., Dong, X., Wang, K., Qi, Y., Chu, C., Zhang, X., Hou, L., 2013. Daidzein induced apoptosis via down-regulation of Bcl-2 / Bax and triggering of the mitochondrial pathway in BGC-823 Cells. Cell Biochem. Biophys. 65 (2), 197–202.

Traganos, F., Ardelt, B., Halko, N., Bruno, S., Darzynkiewicz, Z., 1992. Effects of genistein on the growth and cell cycle progression of normal human lymphocytes and human leukemic MOLT-4 and HL-60 cells. Cancer Res. 52, 6200–6208.

Wu, Y.L., Jiang, X.R., Lillington, D.M., Newland, A.C., Kelsey, S.M., 2000. Upregulation of lipocortin 1 inhibits tumour necrosis factor-induced apoptosis in human leukaemic cells: a possible mechanism of resistance to immune surveillance. Br. J. Haematol. 111 (3), 807–816.

Zafar, A., Singh, S., Naseem, I., 2017. Cytotoxic activity of soy phytoestrogen coumestrol against human breast cancer MCF-7 cells: insights into the molecular mechanism. Food Chem. Toxicol. 99, 149–161.

Zaman, S., Wang, R., Gandhi, V., 2014. Targeting the apoptosis pathway in hematologic malignancies. Leukemia & lymphoma. 55 (9), 1980–1992.

Zheng, W., Sun, R., Yang, L., Zeng, X., Xue, Y., An, R., 2017. Daidzein inhibits choriocarcinoma proliferation by arresting cell cycle at G1 phase through suppressing ERK pathway in vitro and in vivo. Oncol. Rep. 38 (4), 2518–2524.