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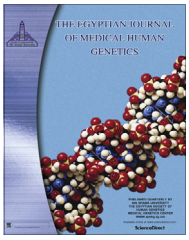

ORIGINAL ARTICLE

Ankaferd Blood Stopper induces apoptosis and regulates PAR1 and EPCR expression in human leukemia cells

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KEYWORDS

Ankaferd Blood Stopper (ABS);
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Abstract *Background:* Ankaferd Blood Stopper (ABS) is a preparation of plant extracts originally used as a hemostatic agent. It has pleiotropic effects in many cellular processes such as cell cycle regulation, apoptosis, angiogenesis, signal transduction, inflammation, immunologic processes and metabolic pathways as well as hemostatic activity. This unique preparation has been widely investigated for its properties. However there are no studies investigating its action on leukemic cells.

Aim: Aim of the study was to examine the ABS action on PAR1 and EPCR in leukemia cells. However, during the experiments, we observed the apoptotic effect of ABS on leukemic cells, particularly Jurkat cells. As a result the mechanism of apoptosis induced by ABS treatment was also explored in the study.

Material and method: Two leukemia cell lines, K-562 and Jurkat, were utilized for the study. Expression analyses of PAR1, EPCR and p21 upon ABS treatment were performed by quantitative real time PCR. Annexin V method was used for apoptosis detection.

Results: Our results demonstrated that ABS alters PAR1 and EPCR expression in K-562 and Jurkat cells in a time and dose dependent manner. Additionally it was found that ABS treatment induces apoptosis in leukemia cells. Possible involvement of PAR1 and p21 in this apoptotic process was observed in Jurkat cells.

Conclusion: This study concludes that depending on the concentration and duration of the application, ABS causes apoptosis by regulating PAR1 and p53-independent p21 involvement in

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apoptosis stimulation in leukemia cells. The composition of ABS plant extracts might be responsible from the apoptotic effect that was observed. We think that our results could contribute to the development of new treatment for leukemia therapy.

1. Introduction

Ankaferd Blood Stopper (ABS) is a preparation of plant extracts composed of *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*. Each of these plants has some effects on the endothelium, blood cells, angiogenesis, cell proliferation and cell mediators [1–5]. ABS originally was known for its hemostatic activity. ABS achieves this effect by inducing the formation of a protein network which includes interactions between ABS and blood proteins, especially with fibrinogen-gamma. Protein agglutination and erythroid aggregation are important components of ABS-induced protein network. Spectrin, ankyrin and actin are vital proteins that are modulated by ABS during erythroid aggregation process [1,40]. ABS also upregulates the level of diverse types of proteins and factors acting on cellular functions such as protein-2 (AP2), androgen receptor (AR), cyclic AMP response element or activating transcription factor-1 (CRE-ATF1), cyclic AMP response element binding protein (CREB), E2F1–5, E2F6, EGR, interferon (IFN)-stimulated response element (ISRE), Myc-Max, nuclear factor-1(NF-1), protein-53 (p53), SMAD2/3, peroxisome proliferator-activated receptor (PPAR) and Yin-Yang (YY1). These factors play a role in various cellular mechanisms, such as cell cycle regulation, apoptosis, angiogenesis, signal transduction, inflammation, immunologic processes and metabolic pathways [1].

Endothelial cell protein C receptor (EPCR) is a member of activated protein C anticoagulant pathway. EPCR shows equal affinity to protein C and activated protein C (APC) [6]. EPCR function has not been fully understood in different cell types where EPCR is known to be expressed, such as hematopoietic cells and cerebral smooth muscle cells. PAR1 is a member of proteinase-activated receptor (PARs) family that is found in seven transmembrane G-protein-coupled receptors group [7]. Activated PAR1 mediates intracellular signaling by coupling G proteins and by using different second messenger systems [8]. Besides its widely accepted role in platelet aggregation and hemostasis, PAR1 has different effects in other physiological functions such as inflammation, Ca (2+) dependent Cl (−) segregation in intestinal epithelial cells, regulation of vascular dynamics, and apoptosis [9–12]. PAR1 involvement in apoptotic process has been shown in neurons, intestinal epithelium and different types of cancer cells by several studies [13–16].

Cell cycle progression is controlled by subsequent activation of cyclin/Cdk complexes. Cyclin dependent kinase inhibitors (CKIs) block the activity of cyclin/Cdk complexes and inhibit cell cycle progression. Overexpression of p21 (Waf1/Cip1), one of the cyclin dependent kinase inhibitors, has an important role in cell cycle arrest and apoptosis [17].

ABS has pleiotropic effects on many types of cells and physiological systems. ABS action on PAR1 and EPCR has been explored in the HUVEC model. These studies shown ABS mediates dose and time dependent, reversible action on PAR1 and EPCR expression in HUVEC cells [18,19]. But to the best of our knowledge, there are no studies investigating the ABS action on PAR1 and EPCR in hematologic malignancies. For this purpose, first we designed a study to analyze the effect of ABS on EPCR and PAR1 in leukemia cells; K-562 and Jurkat cell lines, in different concentrations and at different time points. During the experiments we found that ABS induces apoptosis particularly in Jurkat cells. We then expanded our study to clarify the mechanism of apoptosis induced by ABS and analyzed critical genes in apoptotic pathways such as p21, bcl-2 and bax.

2. Materials and methods

2.1. Ingredients of Ankaferd Blood Stopper (ABS)

An ampoule form of ABS was used in the experiments. Amount of active substances in ampoule form of ABS is as follows: *T. vulgaris* (0.05 mg/ml), *G. glabra* (0.09 mg/ml), *V. vinifera* (0.08 mg/ml), *A. officinarum* (0.07 mg/ml), and *U. dioica* (0.06 mg/ml) [1].

2.2. Cell culture and Ankaferd treatments

Leukemic cell lines, K-562 and Jurkat, were grown in RPMI medium (Lonza, Belgium) supplemented with 20% fetal calf serum (FCS) (Lonza, Belgium), 50 mg/ml penicillin/streptomycin and 1% L-glutamine (Lonza, Belgium). Each cell line was maintained in a humidified incubator at 37 °C supplied with 5% CO_2. Cell lines are from ATCC.

4×10^6 cells from each cell line were counted and seeded into 25 cm^2 flasks within the 5 ml of growth medium. Then ABS was applied into the flasks at two different concentrations, 10 μl/ml (high dose) and 1 μl/ml (low dose). Only ddH_2O (pH: 2), which is similar in pH to ABS, was added to the control flasks. After incubation with ABS at time periods of 20 min, 30 min, 45 min, 1 h, 6 h and 24 h experiments were stopped by removing the ABS containing medium. Cells were washed once with cold 1× PBS and then pelleted by centrifugation to isolate RNA.

2.3. RNA isolation and cDNA synthesis

Total RNA isolations were performed in the MagNA Pure Automated Isolation System (Roche) using MagNA Pure Compact RNA isolation kit (Roche).

cDNA synthesis was carried out using Transcriptor First Strand cDNA synthesis kit (Roche) following the manufacturer's instruction.

2.4. Quantitative PCR

Quantitative PCR (qRT-PCR) was performed using PAR1 and EPCR specific primers and TagMan hydrolysis probes (respectively Universal Probe Library Probes #17 and #50, Roche Diagnostics, Germany) on a Light Cycler 480 II system (Roche). 18S was used for the normalization of PAR1 and EPCR. p21 expression analysis was implemented by using Light Cycler RNA Master SYBR Green I kit (Roche) following the manufacturer's instruction. GAPDH was utilized for the normalization of p21. Primer sequences; PAR-1: F_5′-TCAGAAGATGCCTCCG GATA-3′ R_5′- CACAGA TGGGACAAAGAGTGTC-3′, EPCR: F_5′-GTAGCCAAGACGCCT-3′ R_5′- GATAGGGGTCGCGGA-3′, p21: F_5′-CGATGGA ACTTCGACTTTGTCA-3′ R_5′-GCACAAGGGTACAAGACAGTG-3′, 18S: F_5′- GTAACC CGTTGAAC CCCATT-3′ R_5′- CCATCCAATCGGTAGTAGCG and GAP DH: F_5′-GGCTGA GAACGGGAAGC TTGTCAT-3′ R_5′CAGCCTTCTCCATG GTGGTGAAGA-3′. Each condition of quantitative PCR reactions was studied at least two times. Expression analysis was performed with the Relative Basic Quantification Software, which uses $2^{-\Delta\Delta Ct}$-method, provided with Light Cycler 480 II.

2.5. Detection of apoptosis

Apoptosis detection was performed using Annexin V apoptosis detection kit (sc-4252 AK) (Santa Cruz Biotechnology, Inc.) on live cells according to the manufacturer's recommendations.

2×10^6 cells were counted and seeded into 25 cm^2 flasks. Cells were then treated with ABS in different concentrations and at different time points. The cells were collected by centrifugation at 1500 rpm for 5 min. Cell pellets were washed twice with cold PBS and re-suspended in 1× Assay buffer at concentration of 1×10^6 cells/ml. A 100 µl aliquot of the suspension (containing 1×10^5 cells) was transferred and incubated for 15 min at room temperature with Annexin V-FICT and PI-rhodamine in dark. Cell suspensions were placed on adhesive slides and covered with glass coverslips, followed by immediate observation by fluorescence microscopy and pictures were taken (Nikon Eclipse 80i).

2.6. Statistical analysis

Statistical analysis of the results was obtained by using Excel-one way ANOVA (analysis of variance) test. P values of <0.05 or <0.01 were accepted as statistically significant.

3. Results

3.1. EPCR and PAR1 expression are regulated by ABS in a time and dose dependent manner

To examine whether ABS generates any effect in leukemia cells, two leukemia cell lines were used, the K-562 cell line which is derived from a chronic myelogenous leukemia patient and the Jurkat cell line which is derived from an acute T cell leukemia patient. Cell lines were treated with ABS in various concentrations, 10 µl/ml (high dose) and 1 µl/ml (low dose), and at multiple time points, 20 min, 30 min, 45 min, 1 h, 6 h and 24 h. Then EPCR and PAR1 expressions were analyzed by quantitative PCR method.

As seen in Fig. 1, we observed that the effect of ABS on EPCR is dose and time dependent in both cell lines. In both cell lines, high dose and low dose of ABS affected EPCR expression differentially.

In K-562 cell line, administration of ABS at a low dose caused a decrease in EPCR expression at the beginning. This effect was recovered and passed the control level at 1 h time point. Then a decrease was observed in the expression level, and continued until 24 h. Similarly, at high dose, ABS generated a fast decrease in EPCR expression. Recovery of the expression was observed at 1 h time point and then sharp decrease was followed. Another peak was seen at 24 h (Fig. 1A and B).

In Jurkat cell line, we noticed a similar expression dynamic as observed in K-562 at low dose of Ankaferd treatment (Fig. 1C). But high dose Ankaferd treatment interestingly generated constant decrease in EPCR expression at all of the time points. This decrease was analyzed by one-way ANOVA test and found statistically significant ($p < 0.0001$) (Fig. 1D).

Analysis of PAR1 expression upon ABS treatment also showed, time and dose dependent differential expression patterns in both cell lines (Fig. 2).

In K-562 cells, addition of ABS did not generate any effect at 20 min time point. But at 30 min at both concentrations, decrease in PAR1 expression was observed. After this time point, response to ABS treatment was dissimilar at both concentrations. Low dose of ABS produced an expression increase at 1 h, followed by a decrease at 6 h and an increase at 24 h time point. Conversely, following a similar decrease at 30 min, high dose ABS produced steadily increasing expression until 24 h (Fig. 2A and B).

Jurkat cells, showed a different pattern and more pronounced increases were observed in PAR1 expression after ABS treatment compared to K-562 cells (Fig. 2C and D). When the low dose of ABS was applied, at the beginning (20 min) decrease in PAR1 expression was detected. Then sharp increase (3.5-fold) in PAR1 expression was observed at 30 min. At 1 h the PAR1 expression level decreased to the control level. Another slight increase was observed at 24 h (1.5-fold) (Fig. 2C). Similarly high dose of ABS created decrease in PAR1 expression at 20 min and gradual recovery was seen at 30 min and 45 min. Then much more intense (14-fold) increase in PAR1 expression compared to low dose was observed at 1 h (Fig. 2D). In Jurkat cells, expression changes at both concentrations of Ankaferd treatment (1 and 10 µl/ml) were found to be statistically significant ($p < 0.0001$).

3.2. ABS treatment induces apoptosis in leukemia cells

Anti-neoplastic activity of ABS has previously been reported in osteosarcoma (SAOS-2) and human colon cancer (CaCo-2) cell lines [20,21]. ABS also plays a role in the regulation of some factors which are functional in cell cycle regulation and apoptosis [1]. Additionally, it has been shown that PAR1, depending of the cell type, may induce or prevent apoptosis [12]. In light of this information, during the experiments, we suspected that ABS may induce apoptosis in our cells as well. To clarify this, we performed Annexin V staining which is an established method to detect apoptosis in cells [22].

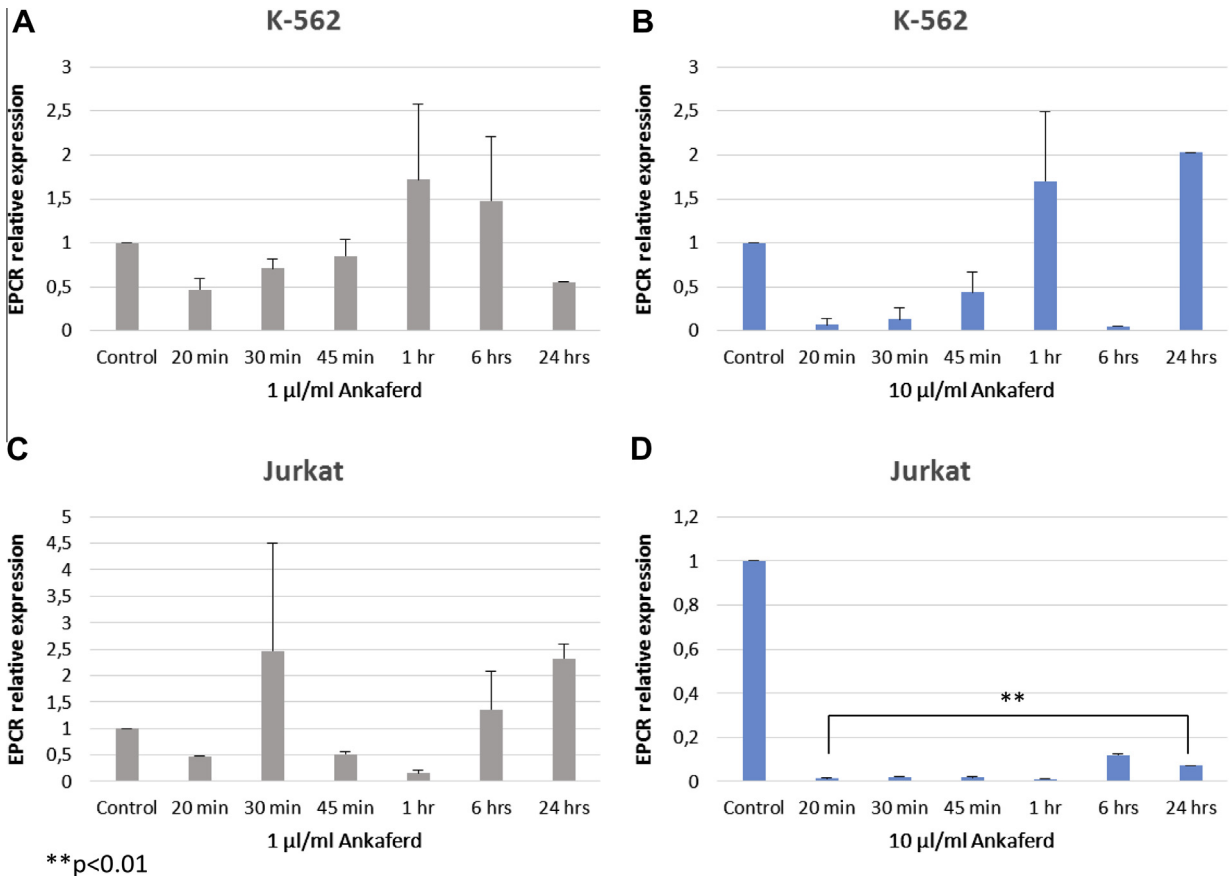

Figure 1 Analysis of EPCR relative expression by qRT-PCR at multiple time points after application of ABS in K-562 and Jurkat cells. EPCR expression in K-562 cells (A) after 1 µl/ml (low dose) of ABS treatment, (B) after 10 µl/ml (high dose) of ABS treatment. EPCR expression in Jurkat cells (C) after 1 µl/ml (low dose) of ABS treatment, (D) after 10 µl/ml (high dose) of ABS treatment. 10 µl/ml ABS treatment caused significant decrease in EPCR expression in Jurkat cells.

Annexin V experiments were implemented using Annexin V apoptosis detection kit (sc-4252 AK) in both cell lines, K-562 and Jurkat, and at four different time points (20 min, 1 h, 6 h and 24 h). Time points we consider as a critical stages, judging by changes we observed in PAR1 expression. ABS was added to the medium and treatments were finalized by pelleting at these four time points. Cells were then labeled with FITC conjugated Annexing V to detect early stages of apoptosis and with PI to detect late apoptosis. Samples were analyzed immediately after labeling under the fluorescent microscope. Meanwhile, light microcopy images were taken subsequent to fluorescent images to visualize cells that were neither stained by FITC nor by PI.

Annexin V staining showed that in K-562 cells low dose of ABS triggers apoptosis at 1 h. But at high dose, profoundly higher apoptosis was observed in 6 and 24 h samples.

In Jurkat cells, low dose of ABS generated the same amount of apoptosis at all-time points. However at high dose, 6 and 24 h samples showed higher levels of apoptosis as compared to other time points (Fig. 3).

3.3. ABS may induce apoptosis via increasing PAR1 and p21 expression in Jurkat cells

To analyze the apoptotic mechanism generated by ABS treatment in leukemic cells, critical genes that are located in apoptotic pathways were investigated. Bax, Bcl-2 and p21 genes were analyzed by qRT-PCR at four different time points (20 min, 1 h, 6 h and 24 h) which were similar in Annexin V staining [23]. Bax and Bcl-2 expression results did not show any changes upon ABS treatment (data were not shown). However, when ABS was applied at different concentrations and at different time points, p21 expression showed interesting changes (Fig. 4). In particular, Jurkat cell line exhibited prominent increase in p21 expression (Fig. 4A). In addition we observed some relationship between PAR1 and p21 expression pattern at similar time points in both cell lines, but especially high dose Ankaferd treatment in Jurkat cell line (Figs. 2D–4A). Addition of high dose of ABS in Jurkat cells produced high increase in both PAR1 and p21 expressions at 1 h, and in parallel we observed more apoptotic cells at 6 and 24 h time points of Annexin V staining at high dose. This finding supports the idea that there is a link between increases in PAR1 and p21 expression and apoptosis that was observed in Jurkat cells (Figs. 2–4).

The effect of ABS on expression changes of PAR1 and p21 was not as strong in K-562 cells. The changes were only one-fold higher in K-562 cells compared to the control. Low dose of ABS treatment generated increase in PAR1 expression at 1 h time point. Similarly Annexin V staining showed more apoptotic cells in 1 h samples at the same concentration. But there was no similar expression increase in p21. In high dose

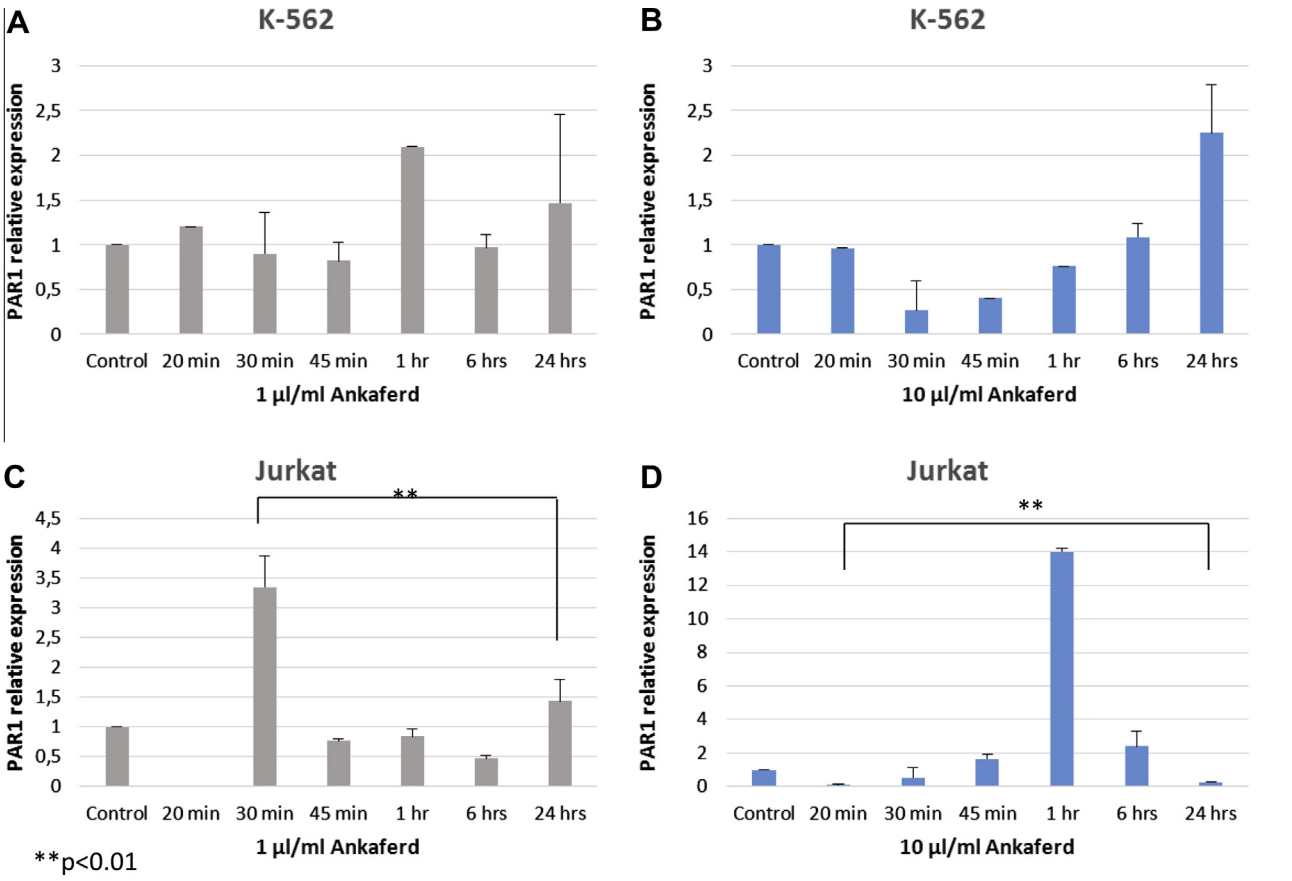

Figure 2 Analysis of PAR1 relative expression by qRT-PCR at multiple time points after application of ABS in K-562 and Jurkat cells. PAR1 expression in K-562 cells (A) after 1 µl/ml (low dose) of ABS treatment, (B) after 10 µl/ml of ABS treatment. PAR1 expression in Jurkat cells (C) after 1 µl/ml (low dose) of ABS treatment, (D) after 10 µl/ml (high dose) of ABS treatment.

of ABS treatment, we observed parallel results with p21 expression and apoptosis increase but not with PAR1 expression (Fig. 4).

It could be deduced from all data that the apoptotic process starts with increase in PAR1 and then p21 expression leading to apoptosis at 10 µl/ml concentration in Jurkat cells which is an acute T cell leukemia cell line.

4. Discussion

Apoptosis or programed cell death is an important cellular protection mechanism which removes the cells that are bearing mutations, DNA damage or showing aberrant proliferation. Different types of stresses such as DNA damage, oncogene expressions, and hypoxia may induce apoptotic signaling pathways. One of the major players of these pathways is p53 tumor suppressor protein [24,25]. Upon activation, by stress stimuli, p53 induces transcription of its target genes and cells undergo either, cell cycle arrest or apoptosis. Activation of p21 through p53 results with cell cycle arrest at the G1 phase [26,27]. But also it has been shown that transcription of p21 by p53 can be repressed by Myc during p53-dependent apoptosis [28]. Conversely there are evidences that p21 also stimulates apoptosis independently from p53 in different types of stresses and diverse cellular contexts [29,30]. Another major checkpoint of apoptosis process is the ratio of pro-apoptotic (BAX) and anti-apoptotic (BCL-2) members. Caspase pathway and mitochondrial dysfunction are located downstream of this checkpoint. Therefore it is important to find the changes of the ratio between these two molecules for the determination of apoptosis [31,32].

In our study, Annexin V results reveal that ABS treatment in Jurkat and K-562 cells cause apoptosis at varying degrees. Apoptosis response was more apparent in Jurkat cells as compared to K-562 cells at both concentration levels. To analyze the mechanism of apoptosis that we observed in leukemia cell lines, Bcl-2, Bax and p21 expression levels of these cell lines were detected by qRT-PCR at both concentrations and different time points. Bcl-2 and Bax expression results did not show any change associated with ABS treatment. However p21 expression changes showed correlations with apoptosis response. Also another striking result was the concordance between PAR1 and p21 expression peaks and apoptosis appearance times. It has been shown that PAR1 can mediate apoptosis in different cell types such as epithelial and endothelial cells, fibroblasts, neuronal cells and tumor cells by either inducing or inhibiting this process [12–16,33]. This dual effect mostly relies on thrombin concentration which is the physiological agonist of PAR1 or other PAR1 activating peptides. It has been shown that low concentrations of thrombin protect tumor cell growth whereas high concentrations of thrombin induce apoptosis [15,33]. Huang et al. demonstrated that in

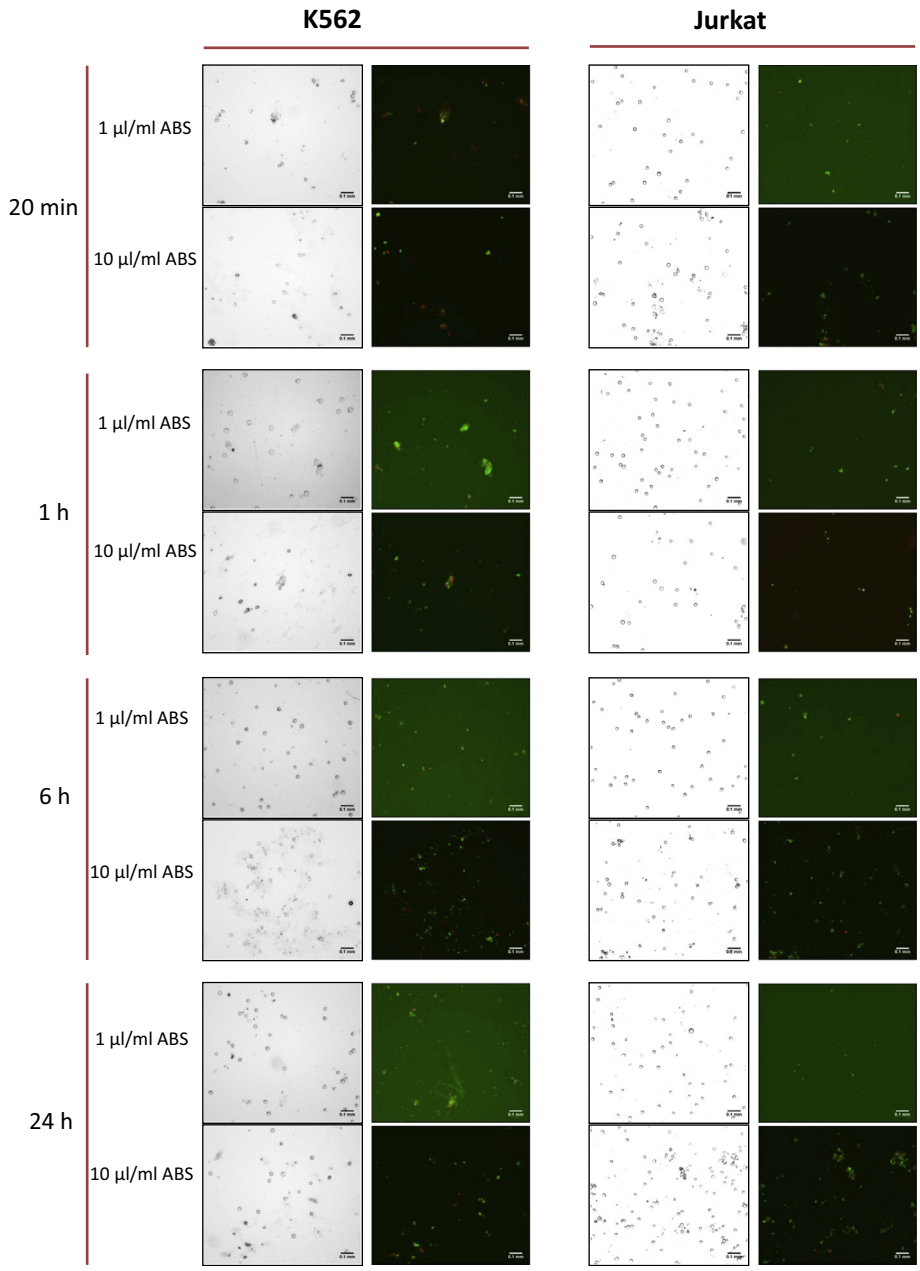

Figure 3 Apoptosis detection by Annexin V staining in K-562 and Jurkat cells after ABS treatment.

several tumor cell line activation of PAR1 by thrombin initiated apoptosis with the up-regulation of p21 and caspases. They also showed that p21 induction is mediated by STAT1-dependent pathway independent from p53 [14]. Our study also showed that, in acute leukemia cells, 10 µl/ml concentration of ABS was able to induce apoptosis process via up-regulation of PAR1 and p21. p53 is mutated in Jurkat cells [34]. Therefore in Jurkat cells up-regulation of p21 is independent of p53. To clarify the involvement of thrombin and other components of the signaling pathway downstream of p21, further analysis is required.

Anti-neoplastic effect of ABS has been explored in solid tumor cell lines such as Saos-2, an osteosarcoma cell line and CaCo-2, a colon cancer cell line [20,21]. In these studies, they observed inhibition in cell proliferation and decline in cell survival as well as loss in their invasion capabilities of these cell lines especially at 10 µl/ml concentration of ABS treatment. In our study, we found anti-neoplastic action of ABS in leukemia cells via apoptosis induction. Apoptosis was more prominent at 10 µl/ml concentration of ABS in Jurkat cells as also were observed in CaCo-2 cells.

Our results also reveal that ABS effects EPCR expression in K-562 and Jurkat cells in a dose and time dependent manner. ABS addition to the cells (in both cell lines) caused decreasing and increasing cycles in EPCR expression. In addition, high dose ABS was able to produce statistically significant constant decrease EPCR expression in Jurkat cell line. These expression differences could be explained by the molecular and genetic

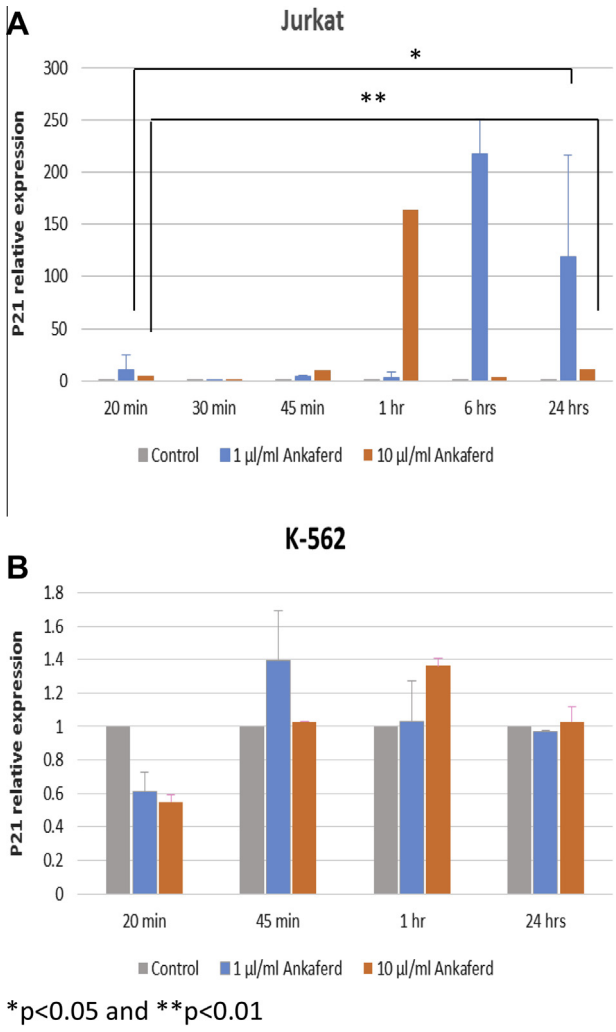

Figure 4 p21 expression changes in leukemia cells after ABS treatment. (A) p21 relative expressions in Jurkat cells, (B) p21 relative expressions in K-562 cells.

differences in cell lines. Additionally decreasing and increasing cycles in EPCR expression in both cell lines were noticeable, which implies that two different factors are possibly acting at these time points. This can be a subject of further studies.

Thrombotic complications are frequently seen in hematological malignancies and solid tumors [35]. Generally, procoagulation factors are thought to be responsible for this complication.

There have been few studies investigating EPCR expression in hematological malignancies [36–38]. In these studies they detected EPCR expression in a wide range of malignant hematological cells. Our study shows EPCR expression in K-562 and Jurkat cells. Time and dose dependent regulation of this expression upon ABS treatment was noticeable. Previously, in HUVEC model, ABS actions on EPCR, PAR-1 and PAI-1 have been investigated where the hemostatic effect of ABS was found to be correlated with the immediate action of ABS via increased expression of prohemostatic PAI-1 and decreased expression of anticoagulant EPCR and PAR-1 [18,19]. Our results were also in parallel with these results, showing that at 10 µl/ml concentration ABS triggers a constant decrease on EPCR expression in acute leukemia cells.

Currently ABS is used for the management of clinical hemorrhages and wound healing. Hemostatic effect of ABS has been tested in the clinical trials and found safe and effective compared to other traditional methods/agents [1,41,42]. Patiroglu et al. used ABS to study its efficacy in oral mucositis of pediatric patients who were treated for hematologic and solid organ malignancies [43]. Their study displayed topical use of ABS in most of these patients causing significant regression in their oral mucositis. Another important point is shown by this study in patients with leukemia ABS used safely without causing leukemic cell proliferation.

Red blood cell (RBC)–fibrinogen interaction and the role of RBCs in the clot formation are very critical hemostatic action of ABS [1]. These factors should also be considered for the development of hemostatic agents in the management of bleeding disorders.

5. Conclusion

Our analyses reveal that ABS responsible for apoptosis induction in different degrees, depending on the concentration and duration of the application by regulating PAR1 and p53-independent p21 involvement in apoptosis stimulation. Additionally ABS treatment produced dose and time dependent changes in the EPCR expression in our leukemia cells.

Disruption of apoptosis generally leads to proliferation of defective cells which ultimately cause oncogenesis. Therefore evasion of apoptosis is accepted as one of the main characteristics of cancer cells [39]. Restoration of deregulated apoptosis in cancer treatment is proposed as an approach for the development of new chemotherapeutics. We think that some of the plant extracts which compose ABS are responsible from apoptosis induction in the leukemia cells. Further investigation could contribute to finding new apoptotic drugs which can be used in leukemia treatment. Also our findings broaden our understanding of mechanism PAR1 and p21 activation and its effect on apoptosis, as well as ABS action on leukemia cells.

Declaration

The authors declare that they have no conflicts of interest.

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