



Hesperidin/Salinomycin Combination; a Natural Product for Deactivation of the PI3K/Akt Signaling Pathway and Anti-Apoptotic Factors in KG1a Cells

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Abstract

AML is a highly aggressive malignant clonal disease of hematopoietic origin. Hesperidin as a polyphenol glycoside, Activates the apoptotic pathway and salinomycin as a k⁺ selective ionophore. We examined how hesperidin and salinomycin induce pro-apoptotic effects in KG1a cells. Cells were divided into four groups; 1) control cells (CTRL), 2) cells treated with hesperidin 85 μM, 3) cells treated with 2 μM salinomycin, 4) cells treated with combination of salinomycin and hesperidin. The MTT assay was implemented to determine the IC₅₀ of hesperidin and salinomycin in KG1a cell lines. Propidium iodide staining and flow cytometry were used to analyze the distribution of the cell cycle. The level of ROS was evaluated by fluorescent microscopy and spectrophotometry. Additionally, Akt, XIAP, Bad, and FOXO1 gene expression was analyzed by real-time PCR. Hesperidin/Salinomycin decreased the viability of KG1a leukemic cells more than Hesperidin and Salinomycin separately. Changes in the shape of apoptotic cells and rise in ROS levels were detected after Hesperidin/Salinomycin treatment. Our findings showed that following Hesperidin/Salinomycin treatment, the expression of PI3K/AKT signaling pathway related genes (AKT, PTEN and FOXO1), were in line with the destruction of KG-1a cells. Furthermore, XIAP and BAD mRNA were regulated to trigger apoptosis in cancer cells. The study discovered that hesperidin and salinomycin, could effectively hinder the PI3K/Akt signaling pathway in leukemia cancer cells. Also, the combination of hesperidin and salinomycin has the potential to be a treatment option for acute myeloid leukemia.

Keywords Acute myeloid leukemia · Salinomycin · Hesperidin · PI3K/Akt signaling pathway · Apoptosis · Cell cycle · Oxidative stress

Introduction

Unregulated growth of hematopoietic stem cells in the bone marrow characterizes AML¹ as a diverse group of diseases [1]. long-term survival rates of the disease is 30% [2]. Malignant cell growth is recognized by the interference of usual intracellular signaling due to mutations or abnormal

external signaling [3]. Intracellular pathways, including the PI3K-Akt² pathway, are aberrantly upregulated in cancers such as AML.

One of the factors in this pathway identified as Akt can operate as a proto-oncogene. Avoidance from apoptosis is a necessary step for cancer tumor advancement [4]. One of the exclusive BH3 proteins, BAD³ plays a role in mitochondrial permeabilization in the apoptotic pathway, which might be over-expressed in cancer cells [5].

FOX⁴ proteins are a superfamily of evolutionarily conserved transcriptional factors that take part in metabolic processes, differentiation, proliferation, cell cycle progression, apoptosis and migration. They inhibit multiple genes involved in cellular processes such as BAD in apoptosis. In

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¹ Acute myeloid leukemia

² Phosphoinositide 3-kinase

³ BCL-2 antagonist of cell death

⁴ Fork head box

this study, we investigate the FOXO1 subfamily. Phospho-FOXO1 Identified in most AML patients due to the phosphorylation of AKT1 signaling [6].

There is another tumor suppressor protein PTEN,⁵ that combats tumor cells and reduces their survival [7]. The important contribution of this tumor suppressor in the progression of myeloid malignancies has been unveiled [8]. Human cancers often display overexpression of XIAP,⁶ which could enable them to evade apoptosis and promote tumorigenesis [9, 10]. One of the drugs that is selected in this study is SAL⁷ which impacts the membrane by acting as an ionophore. It exhibits affinity for alkali ions, particularly for potassium ion [11]. SAL possesses anti-neoplastic features in various human cancers, including hematological malignancies [12]. HESP⁸ (7-rutinoid) is a flavonoid in citrus fruits with anti-tumor features. This natural product Stimulates the apoptotic pathway, which has the potential to assist in fighting cancer cells [13]. So far, no research has been done on the effects of HESP/SAL combination on PI3K/Akt signaling pathway and apoptosis in KG-1a cells, so we studied this issue to make our hypothesis come true.

Material and Methods

Materials

We selected a promyeloblast macrophage cell line known as KG-1a was obtained from the bone marrow of a male White patient, age 59, who was suffering from acute myelogenous leukemia. The Pasteur Institute of Iran (Tehran, Iran) provided KG1a cell lines. Gibco (Life Technologies, USA) provided the RPMI-1640 cell culture medium. Fetal bovine serum (FBS) was obtained from PAN Biowest (France). The culture plates were acquired from SPL (City, South Korea). materials to carry out the cell cycle test, PI-propidium iodide and MTT tests (using DMSO-Dimethyl sulfoxide, 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2-tetrazolium bromide), and also Hoechst 33342, were acquired from Sigma Aldrich (St. Louis, Missouri, USA). Cellular ROS Assay Kit, was provided by Abcam (Japan). The RNA isolation kit was purchased from Sinaclon (Iran). Real-Time PCR requires Real Q Plus 2 × Master Mix Green High ROX™, which was obtained from AmpliQon (Denmark). For cDNA synthesis, the Prime Script™ RT reagent kit (Takara, Japan) was utilized in this study.

⁵ Phosphatase and tensin homolog

⁶ Chromosome-linked inhibitor of apoptosis protein

⁷ Salinomycin

⁸ Hesperidin

Cell Culture

The culture flasks with a capacity of 25 cm² contained acute myeloid leukemia (AML) KG1-a cells which were cultured in RPMI 1640 medium supplemented with 10% FBS and penicillin/streptomycin. The standard conditions for culturing cells included a humidified atmosphere, 5% CO₂, and a temperature of 37 °C.

Determination of Cytotoxicity through MTT Assay

To determine the IC₅₀ value in cells and study the impact of HESP and SAL on cancer cell viability, the MTT⁹ colorimetric assay was conducted. HESP (concentrations ranging from 10 to 150 μM) and SAL (concentrations ranging from 1 to 10 μM) were used to treat KG1a cells and (2 × 10⁴) cells in each well of 96-well plates for 24, 48 and 72 hours were cultured. Following the IC₅₀ calculation, another MTT experiment was done to study the effects of HESP/SAL combination on KG1a cells. Following the assay, 20 μl of MTT (5 mg/mL) was added to each well and left to incubate for 4 hours at 37 °C. DMSO solution was added after the contents of each well were drained. After shaking the plate slowly for 15 minutes, the absorbance was measured at 570 nM with a multi-well plate reader (Quant BioTek Instruments, USA) [9, 10]. Using GraphPad Prism 9.0.0 software (Graph Pad Software, CA, USA), the IC₅₀ values were calculated from the sigmoidal dose-response graph.

Analysis of the Morphology of Apoptotic Cells Using Hoechst 33258 Staining

First KG1a cells were divided to four treatment groups (Control, 85 μM HESP, 2 μM SAL and SAL/HESP). 12-well plates seeded with 5 × 10⁵ KG1a cells for 48 hours. The next step was to harvest the cells and rinse them with cold phosphate-buffered saline (PBS). Next, cells were treated with Hoechst 33258 solution (1 mg/mL in ddH₂O) and the final concentration was adjusted to 100 μg/mL. The cellular morphology was examined using fluorescence microscopy (EUM-5000 FLCD, Labex Instrument).

Cellular ROS Detection Assay

At the beginning of the process cells were placed on a 96 well plate and incubated to attach overnight. In the following stage, cells were treated for 48 hours. Next, the cells were rinsed in 1X Buffer and stained with 25 μM DCFDA¹⁰ in 1X Buffer for 45 min at 37 °C for triple time. After wards

⁹ 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide

¹⁰ Dichlorodihydrofluorescein diacetate

washing with 1X Buffer was executed repeatedly between staining steps. Ultimately, cells were washed in 1X Buffer and changes were determined as a percentage of control after background subtraction. The nucleus of the cells was stained with DAPI¹¹ as well. The cells were observed under microscope for DCF fluorescence at the excitation and emission wavelengths of 535/485 nm (green filter) and for DAPI fluorescence at the excitation and emission wavelengths of 457/357 nm (blue filter). the fluorescence emissions were evaluated by the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The provided data were analyzed using FlowJo software (Ashland, OR, USA).

PI Staining and Flow Cytometry-Based Analysis of Cell Cycle Distribution

PI staining method was utilized to estimate the distribution of cells across different cell cycle phases. For each of the four cell groups, 5×10^5 cells were cultured using this method. Following treatment for 48 hours, the cells were exposed to PI in a darkened room for 30 minutes (PI dosage of 50 $\mu\text{g}/\text{mL}$, RNase A dosage of 100 $\mu\text{g}/\text{mL}$, and 0.5% Triton X-100). During the next stage, Flow cytometry (BD Biosciences, San Jose, CA, USA) was used to analyze the stained cells. The sub-G1, G0/G1, S, and G2/M phases cell distributions were displayed as DNA histograms. FlowJo software from Ashland, OR, USA was used to evaluate the cell cycle dispersal.

Real-Time PCR¹² Analysis of Gene Expression

The three treated and control cell groups of KG1a cells were collected after 48 hours of exposure. RNA extraction was performed using RNX-plus reagent (Cinnagen, Iran). The RNA quality and quantity were evaluated using 1% agarose gel electrophoresis and NanoDrop (Thermo Scientific, USA). To synthesize cDNA, one microgram of total RNA was treated with the Prime ScriptTM RT Reagent Kit (Takara Inc., Kyoto, Japan). The Rotor-Gene Q real-time PCR cyclers (Qiagen) was used to perform triplicate reactions, employing Master Mix Green High ROXTM (Ampliqon, Denmark). ACTB (β -actin) was employed as the housekeeping gene to normalize the expression levels of AKT, PTEN, FOXO1, BAD and XIAP. Gene expression was compared between four cell groups. The relative mRNA expression was evaluated using the comparative $\Delta\Delta\text{Ct}$ method [11]. The real-time PCR primers were synthesized by Oligo (Macrogen, Seoul, South Korea). The Table 1 contains the primer sequences. The following pattern was used to repeat the

Table 1 Sequences of primers utilized in q-PCR

Gene	Primers (5'-3')	Annealing Temp (°C)
Foxo1	F: GCGGGCTGGAAGAATTCAAT R: TCCAGTTCCTTCATTCTGCA	60
PTEN	F: CACACGACGGGAAGACAAGTTC R: TCCTCTGGTCTGGTATGAAGAATG	60
AKT	F: TCTATGGCGCTGAGATTGTG R: CTTAATGTGCCCGTCTTGT	60
XIAP	F: TCCAGAATGGTCAGTACAAAGTTG R: TTTGTTGAATTTGGGAAATTCCT	60
BAD	F: GAGTGAGCAGGAAGACTCCAGC R: TCCACAACTCGTCACTCATCC	60
Beta actin	F: TCAGGGTGTGATGGTTGGTAT R: TCATTGTAGAAAGTGTGGTGCC	60

cDNA synthesis cycles: 95 °C for 30 seconds, 58–64 °C for 30 seconds, 72 °C for 30 seconds, and a final extension of 72 °C for 5 minutes. To test amplification specificity and the existence of primer dimers, a melting curve analysis was conducted on each run. Each primer's mean amplification efficiencies were also calculated by LinRegPCR 2017.9 version [12].

Statistical Analyses

The experiments were conducted in triplicate and expressed as the mean \pm SD. Graph Pad Prism software version 9.0.0 (Graph Pad Software, CA, USA) was utilized to conduct the IC₅₀ calculations. In addition, statistical analysis was performed using an independent sample t-test or one-way ANOVA, with a Tukey's post hoc test conducted via SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). The results were considered statistically significant at P-values of 0.05 (*), 0.01 (**); 0.001 (***); 0.0001(****).

Results

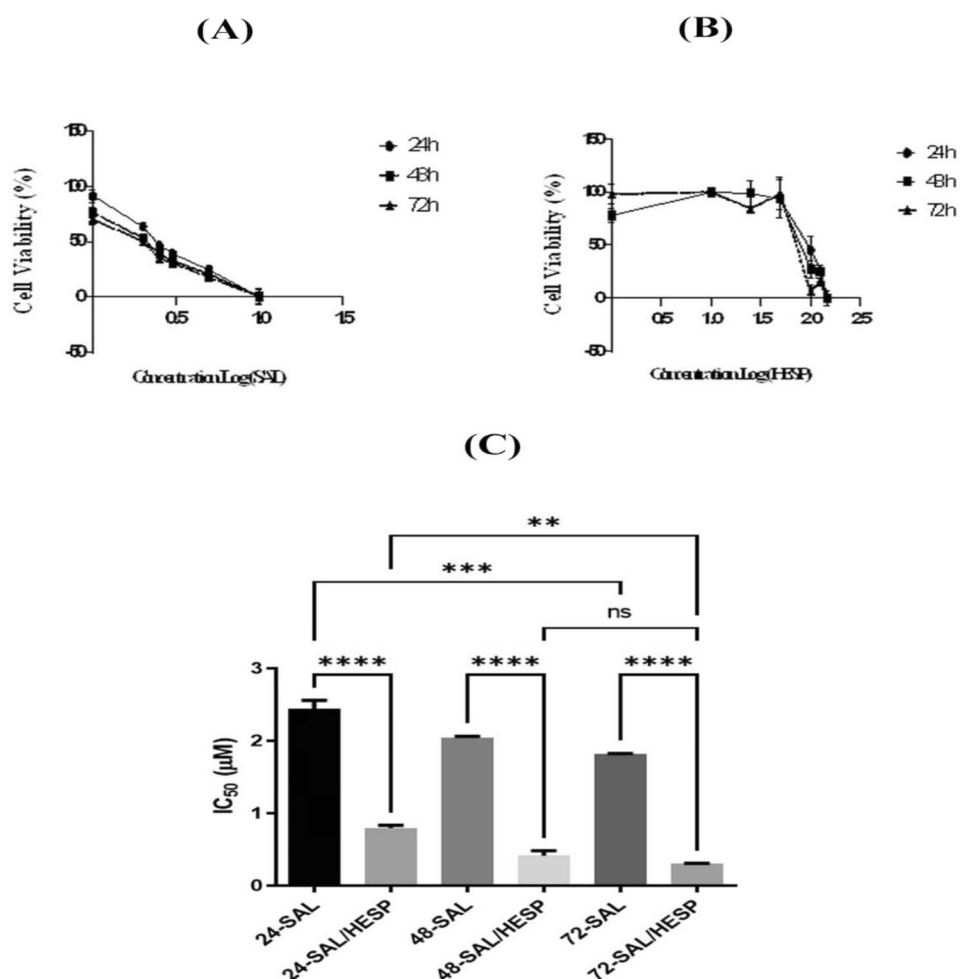
The Viability of KG1a Leukemic Cells Was Reduced by the SAL/HESP Combination

Initially the IC₅₀ of SAL was calculated and the 2 μM for 48 hours was selected (Fig. 1A). In the next stage the selected IC₅₀ for HESP was 85 μM for 48 hours (Fig. 1B). Eventually the effect of SAL/HESP combination on cancer cell viability was assessed by MTT assay. The doses 85 μM HESP and 2 μM SAL were used in preparation of the combination drug. The results showed that the SAL/HESP combination has far pernicious effects than SAL and HESP separately (Fig. 1C).

¹¹ 4',6-diamidino-2-phenylindole

¹² Polymerase chain reaction

Fig. 1 The viability of KG1a leukemic cells was reduced by the SAL/HESP combination. The selected KG1a cells were treated with concentrations of SAL ranging from 1 to 10 μM . These cells demonstrated different growth rates after subjection to SAL (Fig. 1A). The data revealed that after HESP treatment, KG1a viability decreased in dosage or time-dependent manner (Fig. 1B). KG1a cell viability was decreased with SAL/HESP combination (IC_{50} of each drug was combined) in comparison with SAL and HESP separately (Fig. 1C). The results demonstrated as the mean \pm SD of three separate trials. (** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$)



Morphology of Apoptotic Cells Changes after SAL/HESP Treatment

To validate that the sub-G1 fraction is due to apoptosis, a nuclear condensation assay was conducted using Hoechst 33342 staining according to the experimental protocols. Cells are in their steady state without any treatment (Fig. A). Also, SAL, HESP and SAL/HESP was applied to KG1a cell culture at their IC_{50} concentration and the cell death was increased respectively (Fig. B, C, D).

ROS Level Increased after SAL/HESP Treatment

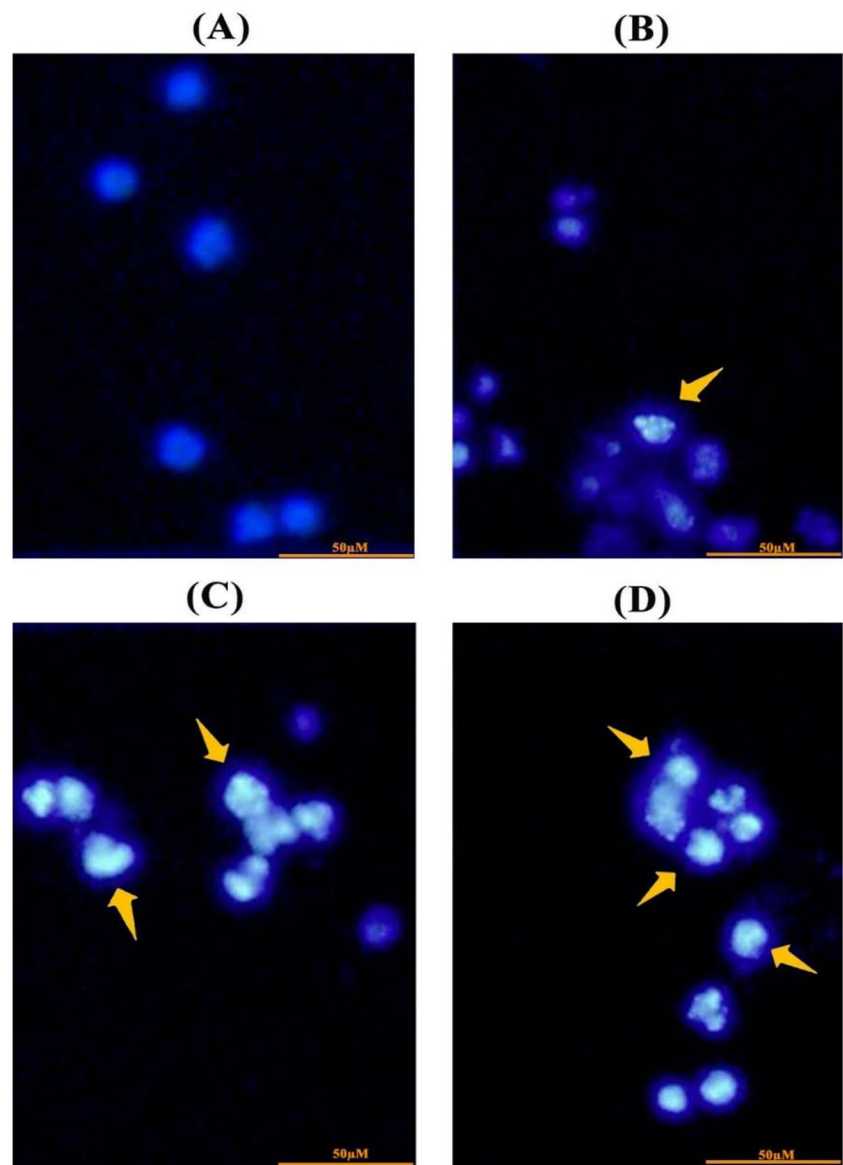
To explore whether SAL and HESP triggered the production of ROS, the total ROS level was assessed using DCFH-DA through flow cytometry. According to the results, there was a gradual rise in ROS production in HESP, SAL, and HESP/SAL respectively in the KG1a cell line. The statistically significant improvement was observed in the HESP administered group after 48 hours (**** $P < 0.0001$) with a 2.7-fold increase. The KG1a cells treated with SAL showed a 1.7-fold surge after 48 h

(* $p < 0.05$). Furthermore, the SAL/HESP group exhibited a 4.1-fold growth (**** $P < 0.0001$) in comparison to the control group after 48 hours of incubation (Fig. 3).

SAL/HESP Caused Sub-G1 Accumulation in KG1a

The proportion of cell cycle phases is shown in KG1a control cells (Fig. 4A). Our research revealed that HESP treated cells were distributed 36.39, 36.57, 24.78, and 2.71% in sub-G1, G0/G1, S, and G2/M phases, respectively (Fig. 4B). The proportion of cell cycle phases changed to 47.26, 31.73, 21.01, and 1.54% at sub-G1, G0/G1, S, and G2/M phases, after a 48-hour treatment with 2 μM SAL (Fig. 4C). The percentage of the cell cycle phases after SAL/HESP treatment was 48.17, 27.52, 24.97 and 2.45% respectively (Fig. 4D). our results show that the G1 phase arrest has happened after treatment with HESP, SAL and SAL/HESP combination treatment resulted in more cell accumulation in the sub-G1 phase. The information about cell cycle phases (before and after treatment) is comprehensively in the chart (Fig. 4E).

Fig. 2 Morphology of apoptotic cells Changes after SAL/HESP treatment. These findings confirmed the outcomes of the cell cycle assay. Chromatin compaction and nuclear fragmentation assay were performed with Hoechst 33258 staining as explained in the methods to examine morphological alterations on treated cells. The KG1-a cells that received no treatment displayed round nuclei with normal and spindle-shaped cell membranes (Fig. 2A). The SAL, HESP, SAL/HESP treatment respectively caused greater increase in nuclear fragmentation and chromatin condensation, while avoiding necrosis induction. The changes are shown by arrows in Fig. 2B, C, D



Evaluation of Gene Expression Quantity by Real-Time PCR

Following the exposure of KG1a cells to HESP, SAL and SAL/HESP, the gene expression level of BAD, FOXO1, PTEN, AKT and XIAP were measured by real-time PCR. According to our discoveries, after 48 hours of incubation with 85 μM of hesperidin, the expression of Bad was remarkably augmented by about 28.13-fold ($***P < 0.001$) (Fig. 5D). It is apparent that there is a significant upward trend in FOXO1 and PTEN gene expression after treatment with HESP, SAL and SAL/HESP respectively (Fig. 5A, B). Besides, Akt gene displayed the minimum gene expression in HESP group with 0.14-fold smaller amount when compared to the control group ($****p < 0.0001$) (Fig. 5C). The results also revealed that XIAP showed a significant

expression level reduction (0.26-fold) in HESP/SAL group compared to the control ($****p < 0.0001$) (Fig. 5E). The general trend of the graph is decreasing in Akt and XIAP.

Discussion

AML is a malignant disease that progresses rapidly due to the proliferation of immature myeloid leukemia cells [14, 15]. There are several signaling pathways which play role in acute myeloid leukemia. One of the signaling pathways deregulated in AML is the PI3K-Akt pathway which is upregulated in cancerous cells [16]. The PI3K-Akt pathway manages vital functions such as proliferation, differentiation, and survival. [17]. In AML, oncogene activating mutations or inactivation of tumor suppressor genes lead

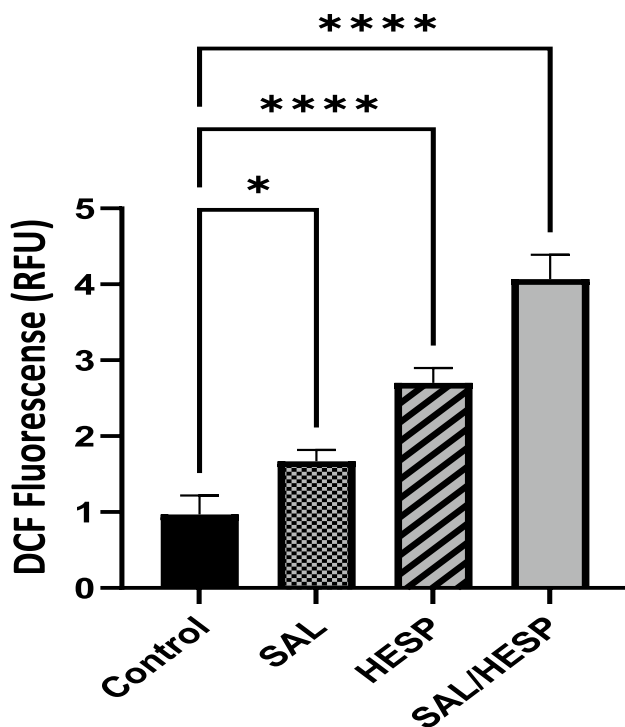
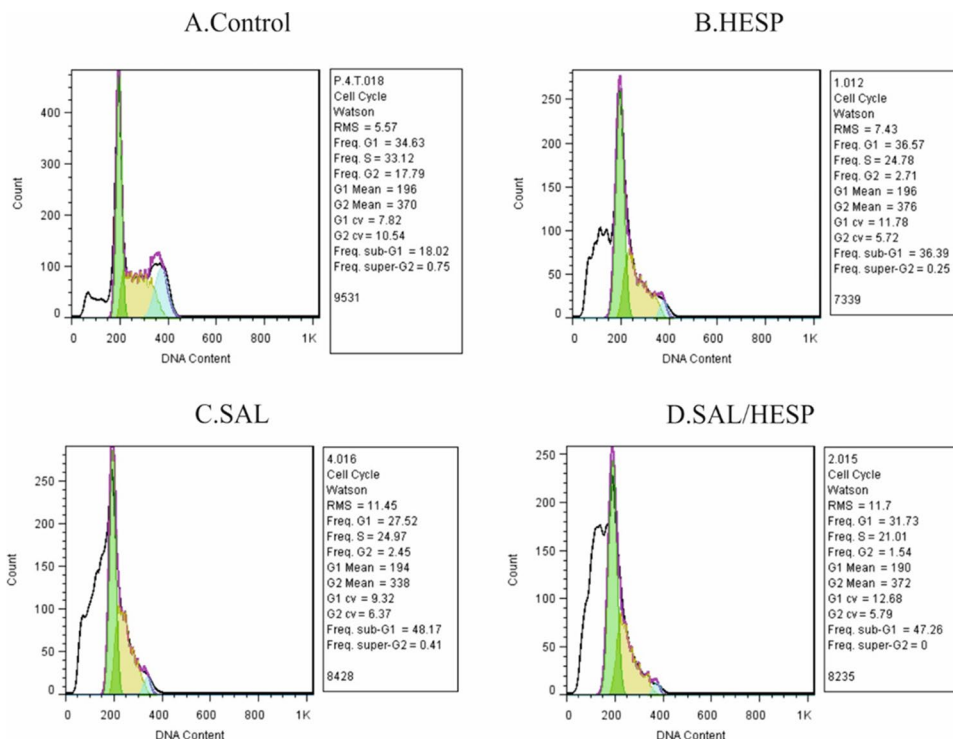


Fig. 3 ROS level increased after SAL/HESP treatment. The difference in ROS folding observed between treated and untreated KG1a cells. The graph shows all data as mean \pm SD ($n=3$). ANOVA was applied to compare the treated and untreated groups (* $p < 0.05$; **** $p < 0.0001$)

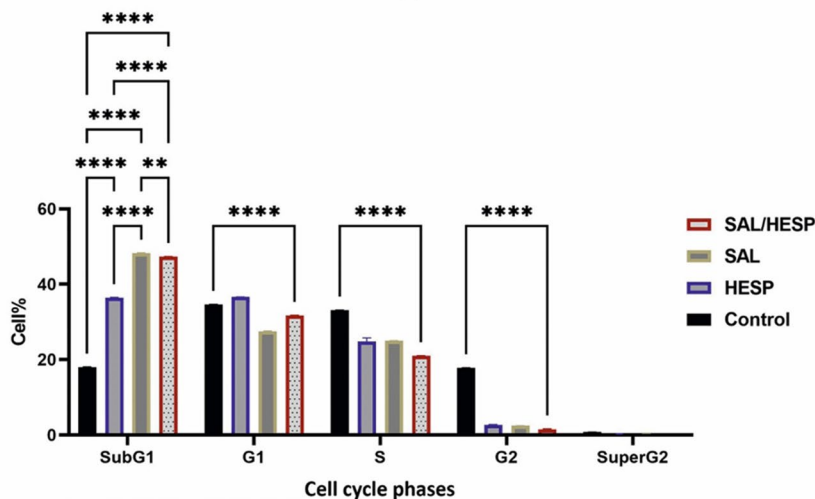
to dysregulation of the PI3K-Akt signaling pathway [17, 18]. There seems to be a connection between the activation of this pathway and decreased overall survival in 60% of AML patients [17, 19]. Akt is a component of the PI3K-Akt pathway, and can function as a proto-oncogene [20]. The findings of our experiment indicated a reduction in akt mRNA expression in KG1a cells following the administration of all three drug categories (SAL, HESP, SAL/HESP), and there was no significant variance among the treatment groups, indicating that the combined therapy of SAL and HESP is not overly effective. Yoon et al. *pointed out that deactivating Akt could potentially increase cancer cells sensitivity to SAL* [21], which was further explored in later studies [22, 23]. The research conducted by Choi et al. suggests that the PI3K/Akt pathway can be targeted by co-treatment with SAL and MK-2206 or LY294002, as potential therapeutic drugs for sensitizing cancer cells [23]. Also Shahbazi et al. showed that Hesperidin suppressed insulin-induced phosphorylation and activation of Akt [24]. Furthermore, Hesperidin treatment reduced the upregulation of Akt and PI3K expression, and effectively preserved the structural integrity of the liver tissues from the development of hepatocellular carcinoma [25]. Additionally, in accordance with the conclusions of another

research, hesperidin hinders breast cancer by blocking the NF- κ B and Akt signaling pathways [26]. Akt can convey an anti-apoptotic signal by phosphorylating downstream target proteins, including Bad and X-linked inhibitor of apoptosis (XIAP), involved in the control of cell growth [27]. Members of the BH3-Only group consist of several pro-apoptotic proteins such as BAD [28]. We assessed the BAD gene's expression and found that HESP was more potent than SAL, and combining the two did not have a synergistic effect on BAD gene overexpression. Savaee et al. revealed that the simultaneous administration of salinomycin/MK-2206 demonstrated a synergistic effect on prostate cancer cell viability and Akt/Bad expressions [29]. Unusual activation of the PI3K/Akt signaling pathway has been documented to potentially serve as a survival mechanism for breast cancer cells by inhibiting apoptosis through Bad protein phosphorylation [30]. Our results are consistent with the findings of Parajuli et al. [31] regarding salinomycin's ability to inhibit Akt and Bad expression in ovarian cancer cells [31]. In our research, PTEN was overexpressed by SAL/HESP compared to the other two treatment groups. PTEN stimulates PIP3 dephosphorylation, resulting in the inactivation of the PI3K-AKT signaling. Hesperidin treatment led to the regulation of the PTEN and decrease in activation of the PI3K/Akt pathway, as noted by Saiprasad et al. [32]. Bonneau et al. detected a partial deletion of the PTEN as a clinically relevant genomic modification in 100% of replicate resistance AML cell line [33]. Another study concluded that the mRNA expression level of PTEN had a remarkable reduction in AML patients. The study uncovered that PPAR γ upregulation was associated with downregulated PTEN in AML patients compared to the control group. Aggerholm's initial investigation revealed that PTEN mutations were present in only 1 out of 59 AML patients, indicating that PTEN's genetic involvement in AML pathogenesis is rare [34]. Similarly, Liu and colleagues, no PTEN mutations were discovered by Liu and colleagues in 62 AML patients, indicating that irregular splicing machinery may target PTEN [35]. In this study, we have surveyed another gene called FOXO1. Important signaling proteins in growth factor signaling, FOXOs are located in the pathway downstream of AKT. AKT activates transcription factors related to survival by phosphorylating FOXO1, 3, and 4 on three different sites, which causes them to translocate into the cell core [36]. all three treatment groups Demonstrated an increase in foxo1 mRNA expression and the variation between these groups was not significant. The uncontrolled expression of FOXO proteins can alter cell destiny and is a crucial factor in numerous cancers, such as leukemia. There are four constituents in this subfamily: FOXO1, FOXO3, FOXO4 and FOXO6. They manage a broad range of cellular functions including apoptosis, DNA damage repair, cell cycle

Fig. 4 SAL/HESP caused sub-G1 accumulation in KG1a. Flow cytometry was used to examine the cell cycle in untreated and treated KG1a cells. The proportion of cell cycle in KG1-a control cells (4A). Distribution of cell cycle in KG1-a cells following 48 hours of incubation with 85 μM HESP, 2 μM SAL and SAL/HESP (4B, C, D). The statistical quantification of data was illustrated in the diagram after treating with hesperidin (Fig. 4E). The outcome was assessed by FlowJo software. (***P* < 0.01; *****P* < 0.0001)



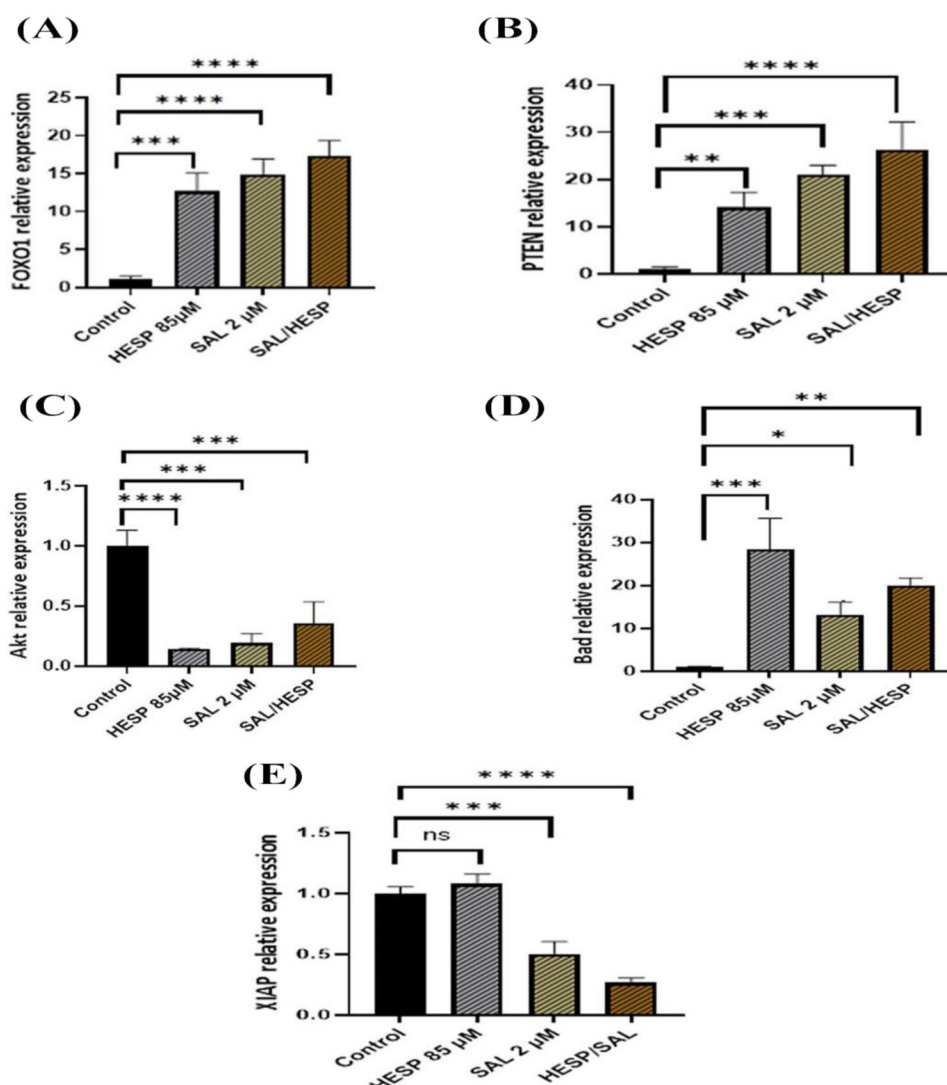
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arrest, and stress response [37]. They trigger or suppress different genes that participate in cellular processes such as BAD in apoptosis. The inactivation of FOXO proteins has been observed in several malignancies, among them being breast cancer, prostate cancer, glioblastoma, and leukemia [6, 38]. The existence of phospho-FOXO1 was discovered to be linked with decreased survival as and a negative unfavorable result in AML patients and it is suggested that phospho-FOXO1 is a significant molecular marker for AML prognosis (63). FOXO1 transcription factor is involved in the regulation of RNOS scavengers, such as superoxide dismutase and catalase, hence its high expression provides protection against oxidative stress

[39]. XIAP was the other tumorigenic factor that we opted for mRNA expression analysis. HESP did not affect on XIAP but its over expression by treatment with SAL and HESP/SAL was significant. Its ability to inhibit activated forms of caspase-3, -7, and -9 is a significant factor in oncogenesis as it prevents cancer cells from undergoing apoptosis [40]. XIAP has been considered an unfavorable gene through a wide range of signal pathways in AML, which includes the caspase pathway and NF-Kb pathway [41, 42]. Suggested by an investigation on 172 primary AML patient samples, there was no relationship between patients and control group [43]. It has been stated that ROS can decrease the expression of XIAP, implying that

Fig. 5 Determination of mRNA expression after exposure to HESP, SAL and SAL/HESP. The q-PCR was utilized to measure fluctuations in transcription levels BAD, FOXO1, PTEN, AKT and XIAP genes. The data is presented as mean \pm SD ($n=3$); The ANOVA compared the treated and untreated group of the cells (Tukey's post hoc test) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$)



inhibiting XIAP can enhance the differentiation of AML cells by up-regulating ROS [44, 45]. Our finding demonstrated that when the cells are exposed to SAL/HESP the ROS is raised considerably and thus may lead to cancer cell death. The consistent oral treatment of hesperidin in rats with breast cancer showed a noteworthy reduction in lipid peroxidation level in the breast cancer animal model [46]. Also, Yu et al. uncovered that Hesperidin induced apoptosis associated with mitochondria in gastric cancer cells and increased the proportion of intracellular ROS [47]. Elevating the levels of hesperidin results in the cessation of the G0/G1 phase of the cell cycle in cervix cancer cells [48], and this result aligns with ours. Also Xia et al. found that treatment of cancer cells with different doses of hesperidin at different times resulted in a significant increase in the percentage of cells in the G0/G1 phase, suggesting the cell cycle arrest at this stage [49].

Conclusion

According to the results of this study, HESP and SAL can be considered as cheap and effective drugs in the treatment of leukemia. In some cases, these drugs can be synergistic and strengthen each other's effects. These natural products make many changes in the expression levels of genes involved in the PI3K/AKT pathway, cell death and oxidative stress in leukemia. More studies in the field of other genes involved in PI3K/AKT pathway as well as their relationship with other signaling pathways such as NF- κ B pathway need further investigations.

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Authors' Contributions Conceptualization: MM
Data curation: MM
Formal analysis: SA
Funding acquisition: MM

Investigation: SA
 Methodology: MM, SA
 Project administration: MM, MN
 Resources: SA
 Supervision: MM, MN
 Validation: SA
 Writing – original draft: SA
 Writing – review & editing: MM, SA.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication All authors consent to the publication of the article in this journal.

Competing Interests The authors declare no competing interests.

Conflict of Interest Not applicable.

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