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Original Article

The cytotoxic activities of the major diterpene extracted from *Salvia multicaulis* (*Bardakosh*) are mediated by the regulation of heat-shock response and fatty acid metabolism pathways in human leukemia cells

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ABSTRACT

Background: Leukemia is one of the most lethal cancers worldwide and represents the sixth-leading cause of cancer deaths. The results of leukemia treatment have not been as positive as desired, and recurrence is common. *Purpose:* Thus, there is an urgent requirement for the development of new therapeutic drugs. *Salvia multicaulis* (*Bardakosh*) is a widespread species that contains multiple phytochemical components with anti-cancer activities. *Methods:* We isolated and characterized the major diterpene candesalvone B methyl ester from S. *multicaulis* and investigated its action as a cytotoxic agent towards sensitive and drug-resistant leukemia cells by the resazurin reduction assay. Additionally, the targeted genes and the affected molecular mechanisms attributed to the potent cytotoxic activities were discovered by transcriptome-wide mRNA expression profiling. The targets predicted to be regulated by candesalvone B methyl ester in each cell line were confirmed by qRT-PCR, molecular docking, microscale thermophoresis, and western blotting. Moreover, cell cycle distribution and apoptosis were analyzed by flow cytometry.
Results: Candesalvone B methyl ester was cytotoxic with IC_{50} values of 20.95 ± 0.15 μM against CCRF-CEM cells and 4.13 ± 0.10 μM against multidrug-resistant CEM/ADR5000 leukemia cells. The pathway enrichment analysis disclosed that candesalvone B methyl ester could regulate the heat-shock response signaling pathway via targeting heat shock factor 1 (HSF1) in CCRF-CEM cells and ELOVL fatty acid elongase 5 (ELOVL5) controls the

Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ALL, acute lymphoblastic leukemia; CAR, chimeric antigen receptor; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; DMSO, dimethyl sulfoxide; ELOVL5, elongase of very long chain fatty acid 5; FBS, fetal bovine serum; GA, genetic algorithms; HRP, horseradish peroxidase; HSF1, heat shock transcription factor 1; IC_{50}, half-maximal inhibitory concentration; IPA, ingenuity pathway analysis; LRP, lung resistance protein; MDR, multidrug resistance; MRP1, multidrug resistance-related protein; MST, microscale thermophoresis; PI3K/AKT, phosphatidylinositol-3-kinase/protein kinase B; PI, propidium iodide; PEA, pathway-enrichment analysis; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation; SKP, Saint Katherine Protectorate.

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fatty acid metabolism pathway in CEM/ADR5000 cells. Microscale thermophoresis showed the binding of candesalvone B methyl ester with HSF1 and ELOVL5, confirming the results of molecular docking analysis. Downregulation of both HSF1 and ELOVL5 by candesalvone B methyl ester as detected by both western blotting and RT-qPCR was related to the reversal of drug resistance in the leukemia cells. Furthermore, candesalvone B methyl ester increased the arrest in the sub-G1 phase of the cell cycle in a dose-dependent manner from 1.3 % to 32.3 % with concomitant induction of apoptosis up to 29.0 % in CCRF-CEM leukemic cells upon inhibition of HSF1.
Conclusion: Candesalvone B methyl ester isolated from *S. multicaulis* exerted cytotoxicity by affecting apoptosis, cell division, and modulation of expression levels of genes contributing to the heat stress signaling and fatty acid metabolism pathways that could relieve drug resistance of leukemia cells.

Introduction

Leukemia is one of the most common types of cancer worldwide, and it was among the leading cause of cancer-related mortality in 2018. It is characterized by the uncontrolled increase of the leukocyte counts in blood circulation or bone marrow (Bray et al., 2018). Globally, it was estimated that 474,519 leukemia cases were diagnosed in 2020 which represents about 3.4 % of all new cancer cases, and about 23,000 patients die each year from leukemia (Sung et al., 2021; Lin et al., 2021). This hematopoietic malignancy affects both children and adults and is more common in males than in females (Saedi et al., 2014). Leukemia can be classified into two main categories, acute and chronic, according to the speed of disease progression and into major four types according to the pathological features: acute myeloid leukemia (AML) and its sub-type acute promyelocytic leukemia (APL), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL) (Mohammadian et al., 2018). Due to this variety of leukemia forms, the outcome and survival of patients depends on the therapeutic approaches. The five-year survival rate of leukemia patients in industrialized countries was 31 %, whereas it was 15 % in developing countries. This highlights the impoverished world's lack of access to high-tech therapy (Allemani et al., 2015). The common treatments used to fight leukemia include combination chemotherapy (cyclophosphamide, fludarabine, prednisone, chlorambucil, and doxorubicin), radiation, targeted therapy, immunotherapy, bone marrow transplantation, and chimeric antigen receptor (CAR)-T cell therapy. Although chemotherapeutic drugs represent the first-line regime for intensive clinical treatment of leukemia patients, there are many complications affecting the outcome, survival, expanding the overall life expectancy of patients besides the relapse of disease as a consequence of the development of drug resistance (Maher et al., 2021; Shi et al., 2019).

The barrier to successful therapy of majority of leukemia is multidrug resistance (MDR) by specific mechanisms such as: i) over-expression of drug efflux pumps (P-glycoprotein, multidrug resistance-related protein (MRP1), lung resistance protein (LRP)); ii) anti-apoptotic pathways and DNA repair mechanisms; iii) alterations of drug targeting genes; iv) upregulation of the PI3K signaling pathway; v) elevated expression of heat shock transcription factor 1 (HSF1); vi) metabolic changes and differential dependency to glucose; vii) biosynthetic blocks; vii) as well as modifications of the tumor microenvironment (Zhang et al., 2019; Mathieu et al., 2019).

Due to the emergence of drug resistance and significant side effects associated with current therapies, there is an urgent need to identify novel safer, and more effective anti-tumor medicines to improve survival rates. The majority of clinically established anti-cancer drugs are derived from natural sources. Medicinal plants contain numerous highly effective secondary metabolites with therapeutic potential against many cancer types (Maher et al., 2021; Ekta et al., 2019; Abu-Darwish and Efferth, 2018). Therefore, screening and investigating the anticancer properties of isolated phytochemicals from natural plants and their underlying mechanism of action will support the identification and development of more novel anticancer agents (Jiang et al., 2016).

Salvia is a genus of the Lamiaceae (Labiatae) family of mint flowering plants, which includes over 900 herbaceous and woody plant species. They are found in Eurasia, Central America, and the Mediterranean area. Many *Salvia* species have been used in traditional medicine to cure colds, bronchitis, TB, obesity, diabetes, hemorrhage depression, dementia, and menstruation problems. *Salvia* species include a diverse spectrum of flavonoids, phenolics, vitamin C, terpenoids, and steroids. Most of them exhibit a broad range of medicinal biological activities, including antibacterial, antioxidant, anti-inflammatory, cytotoxic, anticancer, and antiviral properties. There is a clear interest in exploring the anti-cancer effects and mechanisms of terpenes, which are the major secondary metabolites of the genus *Salvia* (Ezema et al., 2022; Hegazy et al., 2018; Topçu et al., 2018; Jiang et al., 2016; Bonito et al., 2011).

S. multicaulis (*Bardakosh*) is one of the most abundant species in The Saint Katherine Protectorate (SKP) (Sinai Peninsula, Egypt) region. The roots of *S. multicaulis* are rich in diterpenoids. Diterpenoids isolated from *S. multicaulis* were cytotoxic towards CCRF-CEM and CEM-ADR5000 leukemia cell lines (Hegazy et al., 2018) and other tumor cell lines and showed significant activity against *Mycobacterium tuberculosis* (Akdeniz et al., 2021; Abdollahi-Ghehi et al., 2019). Because of these inherent properties, it is conceivable that this particular species possesses a promising potential for future development as an anti-cancer agent. Therefore, this study aims to assess the cytotoxic activities of the major diterpene isolated from *S. multicaulis* (*Bardakosh*) and investigate their promising roles in relieving drug resistance in human leukemia cells.

Materials and methods

Plant material

S. multicaulis Vahl. aerial parts were obtained in Egypt's South Sinai. A voucher sample of plant parts was placed at the Egypt's Saint Katherine Protectorate herbarium as well as the National Research Centre herbarium (voucher No. 310; Giza, Egypt). The plant's air-dried aerial parts were taken with the Saint Katherine Protectorate's approval for scientific investigation.

Extraction and isolation

Hegazy et al. (2018) described all extraction and isolation processes for the target chemical candesalvone B methyl ester.

Cell lines

Drug-sensitive human CCRF-CEM lymphoblastic leukemia cells and their multidrug-resistant P-glycoprotein-overexpressing CEM/ADR5000 subline were obtained from Dr. Axel Sauerbrey (Department of Pediatrics, University of Jena, Jena, Germany) and cultured in RPMI 1640 medium (Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen, USA), and 1 % penicillin-streptomycin (v/v) at 37 °C in a 5 % CO_2 incubator under humidity. The multidrug-resistance phenotype has been described (Efferth et al., 2008; Gillet et al., 2004; Kimmig et al., 1990).

Resazurin cytotoxicity assay

To investigate the cytotoxic effect of the isolated major diterpene fraction, candesalvone B methyl ester, on the leukemia cell lines, we performed the resazurin reduction assay (O'Brien et al., 2000) as previously reported by Chio et al., 2022, Hegazy et al., 2021, and Hegazy et al., 2018. Briefly, 10,000 suspended cells were added to each well of 96-well plates. For generating dose-response curves, different concentrations (0.01 – 100 μM) of the isolated aromatic abietane diterpene candesalvone B methyl ester were added into each corresponding well at a total volume of 200 μl and incubated for 72 h Following that, 20 μl of 0.01 % w/v resazurin (Sigma-Aldrich, Taufkirchen, Germany) was added to each well for 4 h and incubated at 37 °C. The reduced form of resazurin (fluorescent dye resorufin) was then measured in each well at a fluorescence excitation wavelength of 544 nm and emission wavelength of 590 nm using an InfiniteM2000 Pro™ plate reader (Tecan, Crailsheim, Germany). Half-maximal inhibitory concentration (IC_{50}) values of the candesalvone B methyl ester compound were calculated from the established dose-response curves fitting the non-linear regression model using GraphPad Prism® software (Version 6.0, GraphPad Software Inc., San Diego, CA, USA). The IC_{50} values were expressed as mean ± standard deviation (SD). The experiment was repeated three times independently with six replicates each. Dimethyl sulfoxide (DMSO) was used as vehicle control.

Microarray profiling

We treated the cells to discover the affected molecular mechanisms attributed to the potent cytotoxic activities of the candesalvone B methyl ester in CCRF-CEM and CEM/ADR5000 leukemic cells for 24 h with the calculated candesalvone B methyl ester IC_{50} doses (20.95 μM and 4.13 μM, respectively). The methodology for total RNA extraction, and microarray profiling using the novel Affymetrix Clariom Arrays were performed at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ, Heidelberg). The bioinformatics data analysis was conducted using Chipster (http:// chipster.csc.fi/), Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA), and pathway enrichment analysis previously by Hegazy et al., 2021.

Quantitative real-time PCR (qRT-PCR)

CCRF-CEM and CEM/ADR5000 cells were treated with candesalvone B methyl ester at a concentration of IC_{50} for 24 h or left without treatment for control. Total RNA was extracted by RNeasy Kit (QIAGEN, Hilden, Germany) followed by measurement of concentration with NanoDrop 1000 (PEQLAB, Erlangen, Germany). The cDNA conversion from isolated RNA was conducted using LunaScript® RT SuperMix Kit cDNA Synthesis Kit (New England Bio Labs, Darmstadt, Germany). The pairs of primers were formed at the software of https://www.ncbi.nlm.nih.gov/nucleotide/, proven at https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/, and synthesized by Eurofins Genomics (Ebersberg, Germany). Table 1 shows the primer sequences of selected genes. Gene amplification was performed using DNA-binding dye 5 × Hot Start Taq EvaGreen® qPCR Mix (no ROX) (Axon Labortechnik, Kaiserslautern, Germany). qRT-PCR was carried out on CFX384™ (Bio-Rad, Munich, Germany). The quantities of mRNA were calculated using $2^{(-\Delta\Delta CT)}$ computations, with GAPDH employed as an internal reference for normalization (Livak and Schmittgen, 2001).

Molecular docking

All molecular docking simulations were performed using the AutoDock4.2.6 program. The MGL tools (version 1.5.7) was used to generate the pdbqt file for the most predicted target genes affected by candesalvone B methyl ester treatment within the genomes of the human CCRF-CEM and CEM/ADR5000 cell lines, *e.g.*, heat shock factor 1 (HSF1) and ELOVL fatty acid elongase 5 (ELOVL5), respectively, based on the AutoDock protocol. The number of generations and population size for molecular docking calculations were set at 27,000 and 300, respectively. For traditional and intensive molecular docking calculations, the maximum number of energy evaluations (eval) and genetic algorithms (GA) run variables were set to 250.

Microscale thermophoresis

The human recombinant heat shock factor protein 1 (HSF1) and very long chain fatty acids elongase 5 (ELOVL5) were purchased from Sino Biological (Eschborn, Germany) and Cusabio Technology (Houston, USA), respectively to perform microscale thermophoresis (MST). The recombinant proteins were labeled with the Monolith Protein Labeling Kit RED - NHS 2nd Generation (MO-L011, Nano Temper Technologies GmbH, Munich, Germany), following the manufacturer's protocols. This labeling process resulted in final protein concentrations of 1 μM. Titrations were performed over a wide concentration range of the compounds, employing 1:1 dilution steps. The ligand and protein were incubated for 30 min at room temperature in an assay buffer (50 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$, 150 mM NaCl, and 0.05 % Tween-20). Measurements were carried out in Monolith NT.115 standard capillaries (MO-K022, Nano Temper Technologies GmbH, Munich, Germany). Signals were measured using Monolith NT.115 instrument (Nano Temper Technologies) under the settings 20 % LED power and 20 MST for ELOVL5 and 30 % LED power and 10 MST for HSF1 . Data fitting and calculation of K_d values were performed NT Analysis 1.5.41. software (Nano Temper Technologies).

Western blotting for semi-quantification of the most predicted targeted proteins

As stated by Hegazy et al. (2021) and Hegazy et al. (2019), western blotting was applied to explore the response of the protein expression levels of HSF1 and ELOVL5 in both cell lines against the treatment with IC_{50}, 2 × IC_{50}, and 4 × IC_{50} of candesalvone B methyl ester as compared to DMSO. After treatment of CCRF-CEM cells and CEM/ADR5000 cells

Table 1
Sequences of primers used for RT-qPCR.

Gene	Forward	Backward
DNAJB1	5′-CGGACGAGGAGATCAAGCGG-3′	5′-CTGGGGCCACTCCCCTTTAG-3′
BCAP20	5′-TTCAGATGCCCTTTCTAAGGCACA-3′	5′-GTTATGGAGCCCAGGTGCGA-3′
FAM107B	5′-ACCTCCCGGAACCATCAAGA-3′	5′-CAAGCTGCTCCAACTTCTGC-3′
PKIB	5′-AGGCTCTCTCCGTGAAGGAA-3′	5′-ACACATGCTGCTTAGGGCTG-3′
AZIN1	5′-GTTCCATCTCCCAACTTGTC-3′	5′-CCTTTCATCTCAGCCGTATTC-3′
TYW3	5′-GCAGTTTCCAGGAAAGCATGGC-3′	5′-AATGCTCCAGGCATTCCGACAC-3′
SPNS3	5′-TTCCTGCTGACTGCGCTGTACC-3′	5′-AGCCAAAGAGGAACCGAGGCAC-3′
ASIC2	5′-AGTCACACTGTGAACGTGCCCC-3′	5′-TCCCACCTGAGCTTGCTGTTCC-3′
HSF1	5′-AGCCTCCCAAAGCCAAGGAC-3′	5′-CCCCAACCAAACCACAGGAC-3′
ELOVL5	5′-CCTGTGCCTCACGAACTGAT-3′	5′-GACCGTGATCTGGTGGTTGT-3′

(1×10^6 cells /well) with different amplified concentrations of candesalvone B methyl ester for 24 h, cells were lysed by RIPA lysis buffer (Thermo Scientific, USA), and the total protein concentrations were measured using NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Further, 30 μg of each protein sample were denatured by boiling at 95 °C for 10 min prior to separation by 10 % SDS-PAGE. The separated protein samples were then transferred to PVDF membranes at 250 mA for 2 h The blocking of the membranes was done by soaking them in 5 % BSA for 1 h afterward. The blocked membranes were incubated with the primary antibodies against HSF1, ELOVL5, and β-actin (Cell Signaling, USA), respectively, overnight at 4 °C. After washing steps, the membranes were incubated with horseradish peroxidase (HRP) -linked anti-rabbit IgG secondary antibodies (Cell Signaling, USA) for 2 h at ambient temperature, then detected with Luminata™ Classico Western HRP substrate (Merck Millipore Darmstadt, Germany). The Alpha Innotech® Fluor Chem Q system (Biozym, Oldendorf, Germany) was used to identify the signals, and the data was analyzed using Image Studio™ Lite software (LI-COR Biosciences, Lincoln, USA).

Detection of apoptosis by flow cytometry

CCRF-CEM and CEM/ADR5000 cells were seeded onto 6-well plates at a density of 1×10^6 cells/ml, and then treated with different concentrations of candesalvone B methyl ester ($0.25 \times IC_{50}$, $0.5 \times IC_{50}$, IC_{50}, $2 \times IC_{50}$, and $4 \times IC_{50}$) for an incubation time of 48 h The cells were then washed and resuspended in 1 ml of cold PBS, and 500 μl 1 × binding buffer, followed by incubation with 5 μl of annexin V/PE and 10 μl of PI (50 mg/ml) (Thermo Fisher Scientific, Germany) for 15 min in the dark. Flow cytometry (BD Accuri C6 cytometer, Becton-Dickinson, Heidelberg, Germany) was used for obtaining the apoptosis histograms. As referred by Hegazy et al. (2021) and Hegazy et al. (2019), the apoptosis histograms allow differentiating between four cell populations: alive cells, cells in early apoptosis (annexin V positive/PI negative), necrotic cells (annexin V negative /PI positive), and cells in late apoptosis or dead cells (annexin V positive /PI positive).

Analysis of cell cycle distribution by flow cytometry

Once one million of each CCRF-CEM and CEM/ADR5000 cells were seeded per well into 6-well plates, the cells were treated with serial doses of candesalvone B methyl ester ($0.25 \times IC_{50}$, $0.5 \times IC_{50}$, IC_{50}, $2 \times IC_{50}$, and $4 \times IC_{50}$) for 48 h Following washing of cells in PBS, the cells were fixed in 70 % ethanol overnight at 4 °C. Subsequently, the cells were resuspended in 1 ml PBS containing 1 mg/ml RNAase (RNAasA, Thermo Scientific, USA) and 50 μg/ml propidium iodide (PI) (Thermo Scientific, USA) and incubated for 15 min in the dark at room temperature. The percentage of cell populations in each phase of the cell cycle was analyzed by a flow cytometer (BD Accuri™ C6 cytometer, Becton-Dickinson, Heidelberg, Germany) (Hegazy et al., 2021, 2019).

Statistical analysis

All resazurin experiments were repeated three times independently with six replicates each. All the statistical calculations were represented as mean ± standard deviation (SD) using GraphPad Prism® software (Version 6.0, GraphPad Software Inc., San Diego,CA, USA). The data considered significant if $p < 0.05$, and highly significant if $p < 0.01$ using the student's *t*-test.

Results

As a follow-up to the investigation that was previously performed and published by us (Hegazy et al., 2018), components of the air-dried aerial parts of *S. multicaulis* Vahl. species were re-isolated, purified, and re-structured by us for the current study. The same characterized six compounds of abietane diterpenoids as before were obtained, which reflects the reproducibility of our experiments.

One of those six isolated compounds was compound no. 6 (candesalvone B methyl ester), which represented the major diterpene. Its structure (Fig. 1) was established and characterized by mass spectroscopy and advanced NMR methods and identified by comparison with previously published spectral data. Accordingly, the isolated target secondary metabolite was named as candesalvone B methyl ester (Hohmann et al., 2003).

The cytotoxic effect of candesalvone B methyl ester on sensitive and doxorubicin-resistant leukemia cells

As shown in Table 2**,** candesalvone B methyl ester appeared to have a cytotoxic activity after 72 h incubation to both leukemia cell lines CCRF-CEM and CEM-ADR5000 with calculated IC_{50} concentrations of 20.95 ± 0.15 μM, and 4.13 ± 0.10 μM, respectively. The estimated IC_{50} doses were calculated from the dose-response curves that were established previously by our team using the resazurin reduction assay and presented in Fig. 1 (Hegazy et al., 2018).

Microarray hybridization and pathway analyses

After treatment of each cell type with the corresponding IC_{50} concentration of candesalvone B methyl ester for 24 h, high-throughput gene expression profiling displayed that 769 genes were deregulated in CCRF-CEM cells. Among them, 352 genes were upregulated, whereas 417 genes were down-regulated compared to the control. At the same time, high-throughput gene expression profiling displayed 405 genes that were deregulated in CEM-ADR5000 cells. There were 169 upregulated genes and 236 downregulated genes among them. Fig. 2 depicts the expression level for the top 10 up- and downregulated genes of both cell types based on their fold-change values.

Pathway-Enrichment Analysis (PEA) and Boolean network modelling were used to undertake a comprehensive dissection and mining of the candesalvone B methyl ester target-function relationships. In CCRF-CEM and CEM/ADR5000 cell lines, a Voronoi tree map of candesalvone B methyl ester top targeted pathways impacted by the top 20 DEGs (10 upregulated and 10 downregulated genes) targets affected by candesalvone B methyl ester was exhibited. The microarray results were confirmed by qRT-PCR in both CCRF-CEM and CEM/ADR5000 cell lines on two upregulated and two downregulated genes for each. Fig. 2 shows that the treatment with candesalvone B methyl ester upregulated the expression of *DNAJB1* and *BCAP29* while downregulating *FAM107B* and *PKIB* in CCRF-CEM cells. The results exerted upregulation of *AZIN2* and *TYW3* and downregulation of *SPNS3* and *ASIC2* in CEM/ADR5000 cells. The Pearson correlation test revealed the conformity of fold-change between qRT-PCR data and microarray results with *r*-values of 0.989 and 0.998 in CCRF-CEM and CEM/ADR5000 cells, respectively.*HSF1* and *ELOVL5* were two of the most impacted genes in the human genome by candesalvone B methyl ester treatment in CCRF-CEM and CEM/ADR5000 cells, respectively (Fig. 3).

Molecular docking

A molecular docking approach was applied to confirm the molecular interaction between the isolated candesalvone B methyl ester molecule and the HSF1 and ELOVL5 proteins at the atomic level. The calculated energy of the binding affinity between candesalvone B methyl ester and HSF1 or ELOVL5 protein molecule was predicted to be equal to −6.0 kcal/mol (Fig. 4). The docking score proves and emphasizes the probability of regulation and targeting of HSF1, and ELOVL5 proteins by the phytochemical compound candesalvone B methyl ester.

Microscale thermophoresis

We used microscale thermophoresis as a sensitive technology to

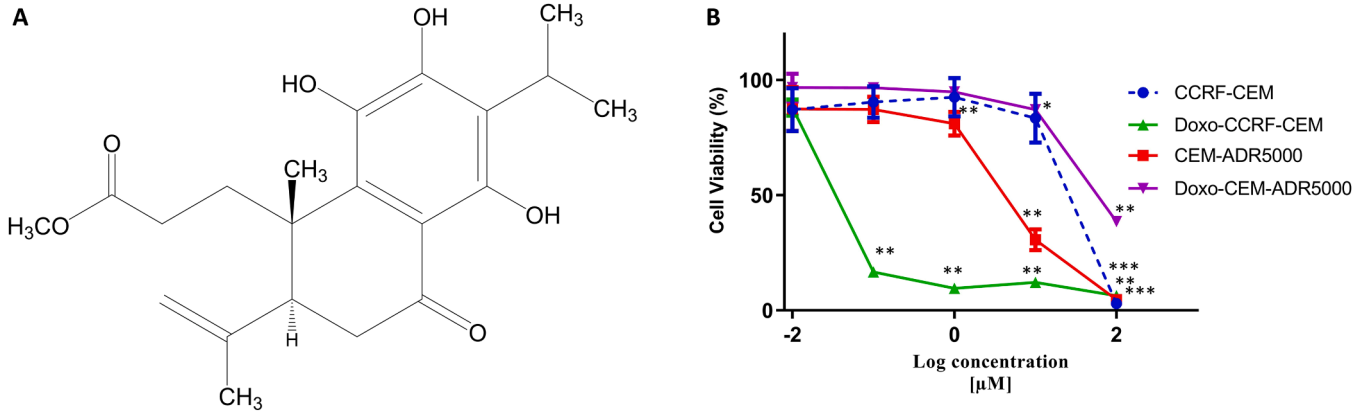


Fig. 1. The structure of candesalvone B methyl ester and its cytotoxic effect. (A) The chemical structure of candesalvone B methyl ester. **(B)** The dose response curves of candesalvone B methyl ester and doxorubicin towards drug-sensitive and drug-resistant cell lines as determined by the resazurin assay. Mean values and standard deviations of each three independent experiments with each six parallel measurements**;** Statistical analysis was done by paired student's *t*-test $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

Table 2
The cytotoxicity of isolated candesalvone B methyl ester toward CCRF-CEM and CEM-ADR5000 human leukemia cells.

Cell line	IC_{50} of candesalvone B methyl ester (µM)
CCRF-CEM	20.95 ± 0.15
CEM-ADR5000	4.13 ± 0.10

The data represents the mean calculated IC_{50} doses ± standard deviation (SD). The experiment was repeated three times independently with six replicates each.

determine the binding affinity between candesalvone B methyl ester and the human recombinant heat shock factor protein 1 (HSF1) and very long chain fatty acid elongase 5 (ELOVL5). For this purpose, the recombinant HSF1 and ELOVL5 were labelled and titrated against various concentrations of the candesalvone B methyl ester. The resulting affinity curves demonstrated strong interactions between the HSF and ELOVL5, with K_d values of 1.16 and 2.04 µM, respectively (Fig. 5).

Western blotting for semi-quantification detection of HSF1 and ELOVL5 protein expression levels

For biological validation of the theoretical computerized predictions that both HSF1 and ELOVL5 are targeted by the active compound isolated from *S. multicaulis*, candesalvone B methyl ester, we analyzed the protein expression levels of HSF1 and ELOVL5 using the western blotting technique. After leukemia cells were subjected to increased

A. CCRF-CEM

Top 10 Up and Down DEG	FCM	P. Value
HSPA1B	4.084049	0.04975
HSPA8	2.751084	0.048298
DNAJB1	2.219139	0.04139
BCAP29	1.815038	0.001542
NAGK	1.741101	0.030906
FAHD1	1.729074	0.049584
AFMID	1.70527	0.042019
TBL2	1.670176	0.040387
CDS2	1.647182	0.039349
OST4	1.624505	0.003898
FAM107B	-2.60268	0.026118
PKIB	-2.49666	0.0174
CTH	-2.37841	0.033401
ANKRD11	-2.36199	0.007417
BATF3	-2.23457	0.00829
LRMP	-2.20381	0.031601
KRT1	-2	0.03468
LONP1	-1.98618	0.013632
GPA33	-1.97247	0.032732
SHISA2	-1.89212	0.034218

B. CEM/ADR5000

Top 10 Up and Down DEG	FCM	P. Value
PDPR	1.464085696	0.010753
ELOVL5	1.453972517	0
RPLP1	1.394743666	0.013996
CTDSPL2	1.347233577	0
AZIN1	1.337927555	0.018888
TYW3	1.319507911	0.020485
ASRGL1	1.319507911	0.04704
MKKS	1.319507911	0.020485
PBX3	1.310393404	0.021315
MTDH	1.310393404	0.048612
RNY4	-1.681792831	0.022686
LOC729683	-1.613283518	0.007146
SPNS3	-1.569168196	0
ASIC2	-1.474269217	0.010753
OPLAH	-1.443929196	0
PPP2R4	-1.394743666	0.042879
SCAMP3	-1.347233577	0.045377
NR0B2	-1.337927555	0.045377
CCL28	-1.337927555	0.018117
DPH2	-1.319507911	0.045377

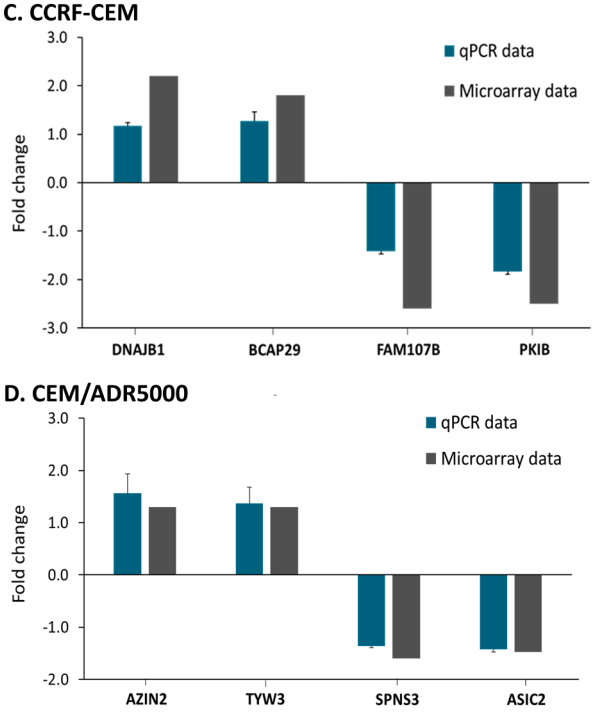


Fig. 2. Transcriptome analysis of leukemia cells treated with candesalvone B. (A and B) Microarray hybridization of both cell lines treated with IC_{50} dose of candesalvone B methyl ester. The top 10 up- and downregulated genes are displayed according to their mRNA expression level. DEG, differential expressed gene; FCM, fold change mean. **(C and D)** validation of microarray hybridization by qRT-PCR**.** Gene expressions are altered upon the treatment with candesalvone B methyl ester in CCRF-CEM and CEM/ADR5000 cells. A strong correlation of fold change was observed between microarray hybridization and qRT-PCR results (R-values of 0.989 and 0.998 in CCRF-CEM and CEM/ADR5000 cells, respectively).

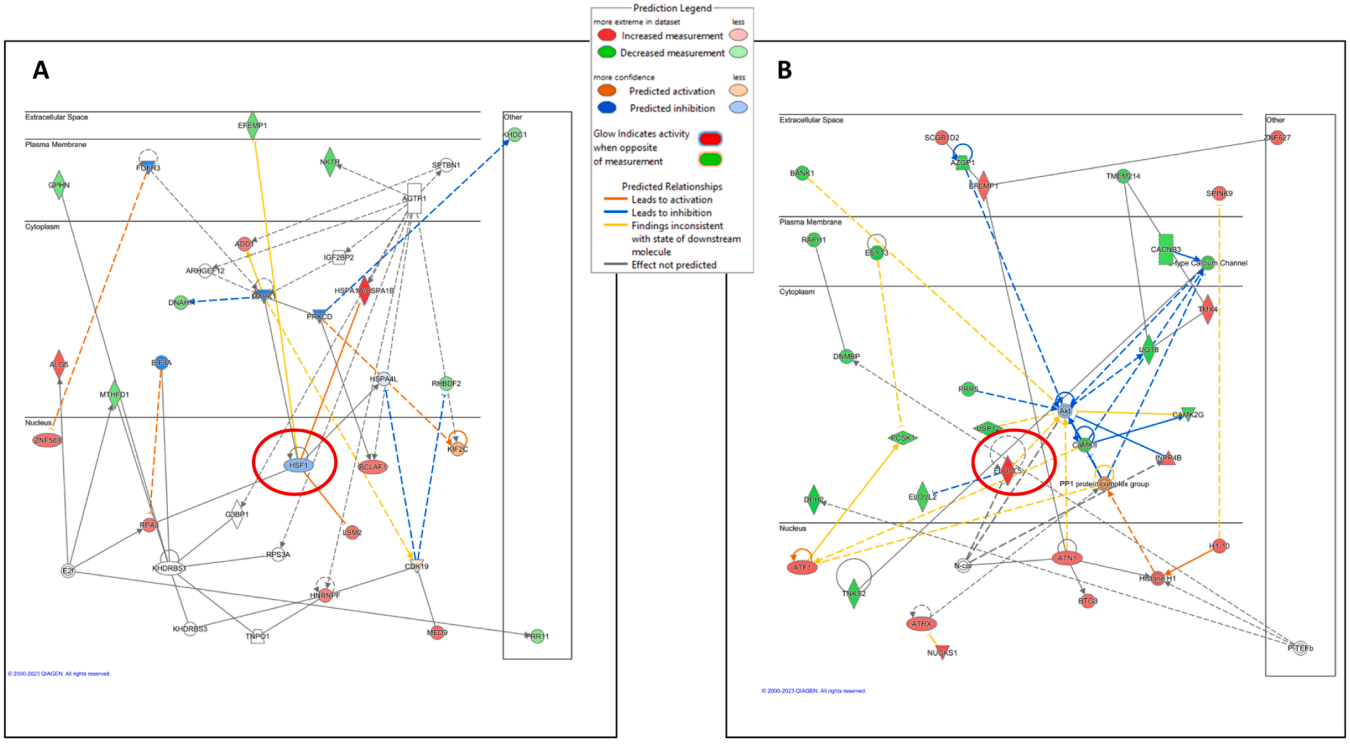
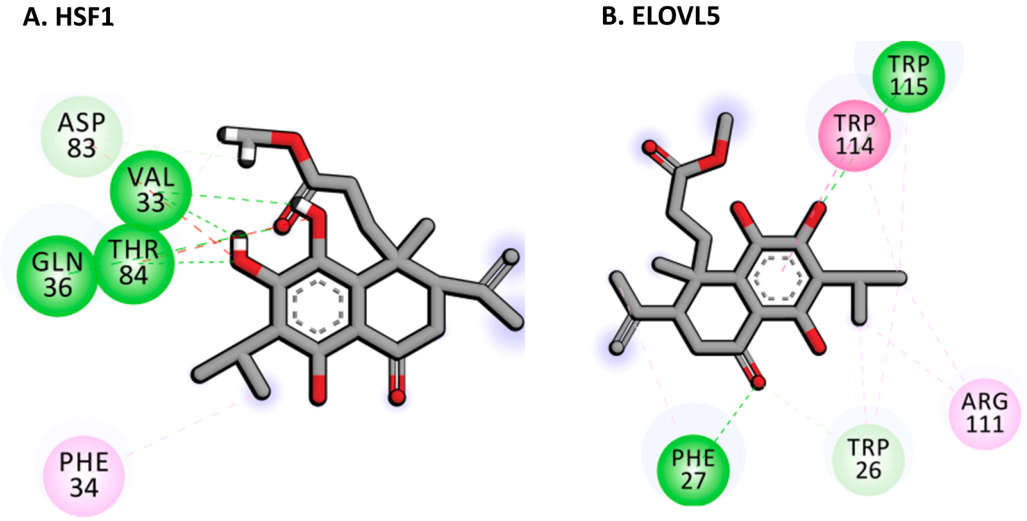


Fig. 3. Molecular network generated by the Ingenuity Pathway Analysis (IPA) tool. (A) the network shows HSF1 as the target gene most affected by candesalvone B methyl ester treatment within the human genome of CCRF-CEM while in CEM-ADR5000 cell lines ELOVL5 appears to be affected as shown in **(B)**.



PHE= phenylalanine, **TRP**= tryptophan, **ARG**=arginine, **GLN**= glutamine, **THR**= threonine, **VAL**= valine, **ASP**= aspartic acid.

Fig. 4. Molecular docking of *HSF1* and *ELOVL5* with candesalvone B. (A and B) Molecular representation of candesalvone B and its interacting amino acids with HSF1 and ELOVL5, respectively.

concentrations of candesalvone B methyl ester for 24 h, the protein expression levels of HSF1 and ELOVL5 were downregulated in response to the higher doses of candesalvone B methyl ester in CCRF-CEM cells and CEM/ADR5000 cells, respectively (Fig. 6).

RT-qPCR validation of HSF1 and ELOVL5 mRMA expression levels

HSF1 and *ELOVL5* mRNA expression levels were analyzed at three doses (IC_{50}, 2 × IC_{50}, and 4 × IC_{50}) of candesalvone B methyl ester after

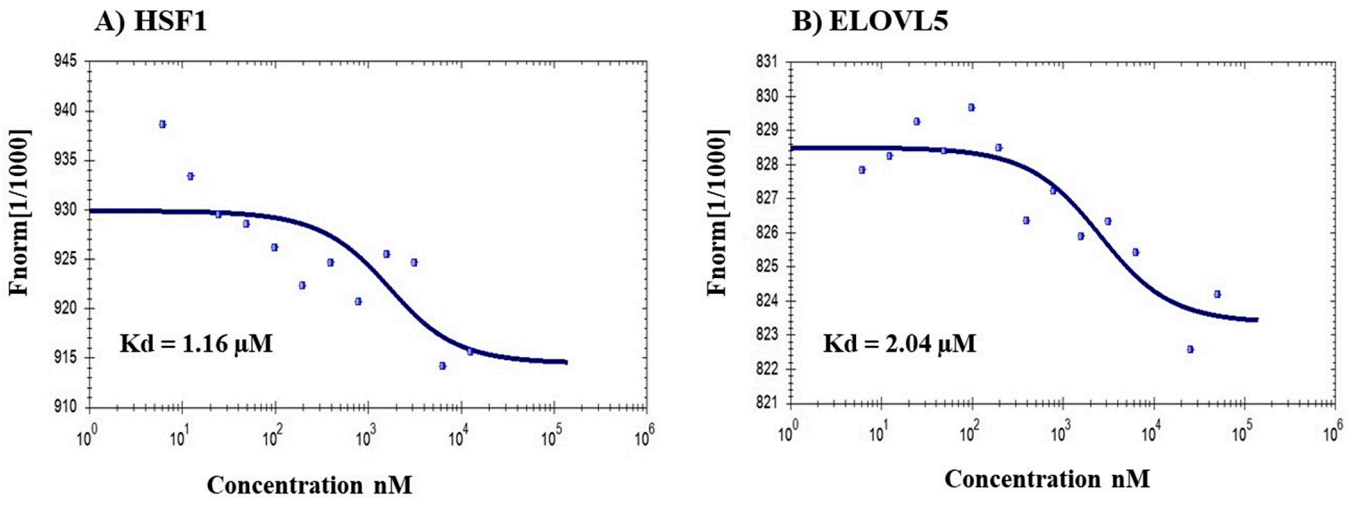


Fig. 5. Microscale thermophoresis binding assay of candesalvone B methyl ester with *HSF1* (A) and *ELOVL5* (B).

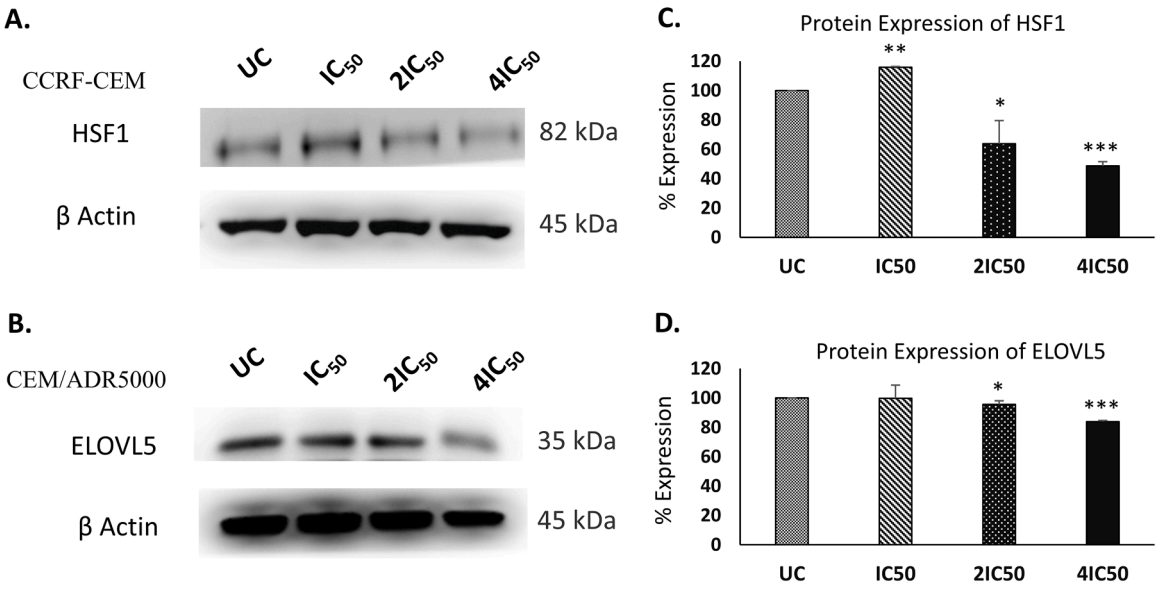


Fig. 6. Western blotting of proteins targeted by candesalvone B methyl ester (A and B). The expression of *HSF1* and *ELOVL5* were decreased in response to increasing IC_{50} doses of candesalvone B methyl ester in comparison to untreated control (DMSO). β-actin was used as the normalized internal control. **(C) Quantification of protein expression of HSF1 and ELOVL5 in response to increasing concentrations of candesalvone B methyl ester.** The data were represented as mean ± SD. Each experiment was repeated three times independently.

24 h incubation. Fig. 7 shows the downregulation of the mRNA levels in a dose-response manner. A careful comparison of Fig. 7 with the graphs in Fig. 6 reveals that the patterns of downregulation are similar. While HSF1 protein and mRNA expression levels decrease gradually with each increased dose, ELOVL5 protein and mRNA levels remain similar at the IC_{50} and 2 × IC_{50} doses, and then show increased downregulation at the 4 × IC_{50} dose (Fig. 7)

Induction of apoptosis by candesalvone B methyl ester

By using variable concentrations of candesalvone B methyl ester (0.25 × IC_{50}, 0.5 × IC_{50}, IC_{50}, 2 × IC_{50}, and 4 × IC_{50}), a remarkable elevation in the number of apoptotic CCRF-CEM cells of about 29.0 % was detected after 48 h incubation (Fig. 8). On the other hand, no significant change was observed upon candesalvone B methyl ester treatment of CEM/ADR5000 cells in comparison with control (data not shown).

Analysis of cell cycle distribution

To explore the effect of variable concentrations (0.25 × IC_{50}, 0.5 × IC_{50}, IC_{50}, 2 × IC_{50}, and 4 × IC_{50}) of candesalvone B methyl ester on the percentage of leukemic cells accumulated in each phase of the cell cycle, we used a flow cytometric analysis of the cell cycle distribution pattern. Our results indicated a dose-dependent increase in the population of CCRF-CEM cells in the sub-G1 phase ranging from 1.3 % to 32.3 % upon incubation for 48 h with candesalvone B methyl ester. The percentages of cells in G2/M phases were accordingly decreased (Fig. 9). There was no significant change observed for candesalvone B methyl ester treatment toward CEM/ADR5000 leukemia cell lines as compared to the control (data not shown).

Discussion

Leukemia consists of a set of hazardous subtypes that are the biggest cause of mortality worldwide, and its prevalence is rising in both

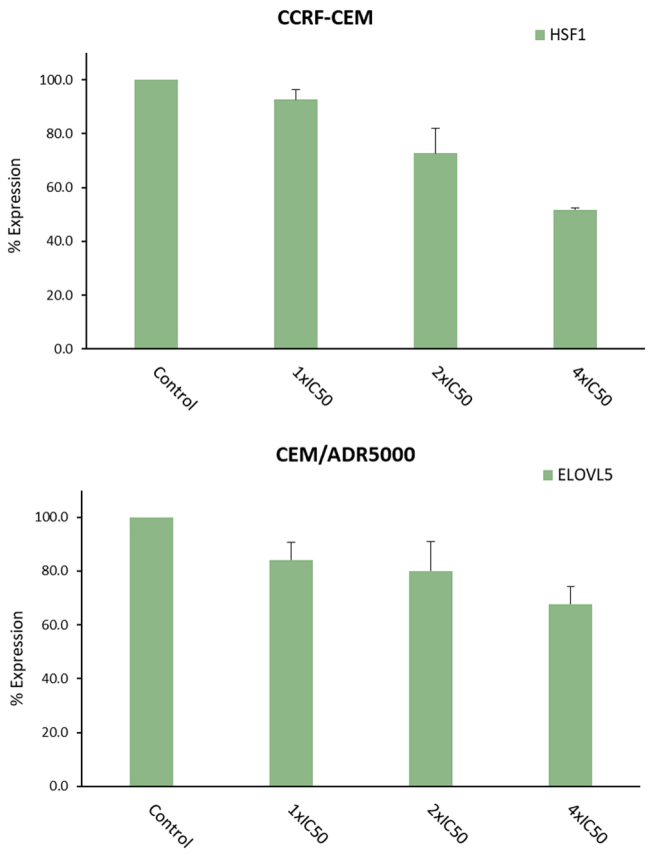


Fig. 7. RT-qPCR analysis of *HSF1* and *ELOVL5* mRNA expression levels upon incubation for 24 h **with candesalvone B methyl ester** (A) A dose-dependent downregulation of HSF1 mRNA expression levels was observed in CCRF-CEM cells. (B) ELOVL5 mRNA expression in CEM/ADR5000 cells was similarly downregulated at both IC_{50} and 2 × IC_{50} doses, with a further downregulation observed at the 4 × IC_{50} dose.

developed and developing countries. Unlike solid tumors, leukemia cannot be managed surgically, but it may be controlled by various therapeutic procedures including intense chemical therapies (Chennamadhavuni et al., 2022). Although some of these techniques are therapeutically helpful, they have the potential to produce major problems in leukemia patients. Similarly, many chemotherapy medications have serious adverse effects, and some cancer cells have acquired resistance to them. As a result, there is an urgent need to develop therapeutically more effective medications that are selectively harmful to cancer cells without hurting normal cells (Ezema et al., 2022).

Compounds isolated and purified from natural plants have been the main source of medicines and drugs, which can be useful in the treatment of wide types of cancers including leukemia (Ekta et al., 2019). The main aromatic abietane diterpenoid fraction employed in this work, candesalvone B methyl ester, was isolated and identified from the air-dried aerial portions of *S. multicaulis* Vahl using dichloromethane/methanol (1:1). The isolated fraction was identified as candesalvone B methyl ester by comparing its characterized structure with previously published spectral data. The methyl ester of candesalvone B was recognized firstly in the methanolic extract of aerial parts of *Salvia candelabrum* by Hohmann et al., 2003. Further anti-oxidative activities of candesalvone B methyl ester counteracted lipid peroxidation (Janicsák et al., 2003). This phytochemical component showed promising cytotoxicity against sensitive and drug-resistant cells, *e.g.*, CCRF-CEM (IC_{50} = 20.95 ± 0.15 μM), and CEM/ADR5000 (IC_{50} = 4.13 ± 0.010 μM) leukemic cells according to our previously published paper (Hegazy et al., 2018), but with unknown molecular mechanisms, or without determination of the mode of action of this obvious cytotoxic activity. Therefore, we were concerned with discovering of the anticancer action mechanisms of the recently isolated methyl ester in leukemia cell types. The cytotoxic activity shown by this diterpene, candesalvone B methyl ester, was indicated to be linked with its pharmacophore structure which is an aromatic C-ring with *para*-and *ortho*--phenolic hydroxyl groups (catechol) together with the characteristic isopropyl moiety (Aoyagi et al., 2006). Moreover, the isolated compound that was used in this research study constitutes of endocyclic α, β-unsaturated carbonyl functional groups located at C-7 on B-ring, which represent an essential structure for enhancing cytotoxic activity against leukemia cells (Dai et al., 2015; Saeed et al., 2015).

As no more informative publications are available concerning this compound, high-throughput gene expression profiling was applied to search for signaling pathways that are regulated by candesalvone B methyl ester fraction and identification of the functional target genes that are affected by the isolated methyl ester. This high-throughput gene expression technology was estimated particularly to resolve the mysterious molecular mechanism of actions of novel cytotoxic phytochemical compounds (Hegazy et al., 2021; Mahmoud et al., 2020). Accordingly, the high-throughput gene expression profiling of this research paper reported that genes contributing to the heat-shock response signaling pathway and fatty acid metabolism pathways were differentially expressed as a response of CCRF-CEM and CEM-ADR5000 leukemia cells, respectively, to candesalvone B methyl ester treatment. By using independent additional approaches such as qRT-PCR, molecular docking, microscale thermophoresis, and western blotting, our results confirmed the involvement of these two pathways in the mode of action of candesalvone B methyl ester via targeting HSF1 (the mediator of heat-shock response pathway in CCRF-CEM cells), and ELOVL5 (the mediator of fatty acid metabolism pathway in CEM/ADR5000 cells) with a binding affinity of −6.0 kcal/mol between investigated compound and HSF1 or ELOVL5 proteins.

HSF1 is a transcription factor that induces the expression of HSPs (heat shock proteins), cytoplasmic chaperones, to buffer and refold the misfolded proteins which were accumulated within cells as a response to various cellular stresses including heat shock, ischemia, and aging (Tchénio et al., 2006). HSF1 also regulates the transcription of genes contributing to cell cycle control, synthesis of protein, embryonic development, and metabolism of glucose (Cyran and Zhitkovich, 2022). As a major regulator of such heat shock response, the elevated expression levels of HSF1 were associated with poor survival, prognosis, and aggressiveness of many tumors such as breast, liver, endometrial, esophageal, oral squamous cell, and ovarian carcinomas. Consequently, high expressed levels of HSPs were reported in many types of cancer and investigated to be involved in tumor formation, poor prognosis, apoptosis inhibition, and drug resistance development (Huang et al., 2021; Yang et al., 2021; De Andrade et al., 2020; Chatterjee et al., 2016). Moreover, the role of HSF1 as an activator of a drug resistance transporter, P-glycoprotein (ABCB1/MDR1), was proven. The activation of the *ABCB1/MDR1* gene by HSF1, in a heat-shock-independent manner, poses a major obstacle to the success of cancer chemotherapeutic agents in many types of cancer cell lines such as liver, prostate, cervical, osteosarcoma, and leukemia. This activation was suggested to be a nuclear event through the modulation of *MDR1* mRNA maturation pathway by HSF1 via acting on splicing or stability of *MDR1* mRNA precursors (Tchénio et al., 2006). Therefore, the targeting of HSF1 and the downstream contributed HSPs will be a good opportunity for discovering novel anti-cancer treatments that will enhance apoptosis by inhibiting related anti-apoptotic proteins and cell cycle arrest which is mediated directly by suppression of cyclin D, as well as, increasing of p21 and p27 levels (Carpenter and Gökmen-Polar, 2019; Dong et al., 2019) besides reverse of the drug resistance phenotype (Yun et al., 2019).

Interestingly, our findings demonstrated that HSF1 is a major predicated target of candesalvone B methyl ester, with a reverse correlation between its expression and the concentration of candesalvone B methyl ester treated compound. The HSF1 expression level was detected by

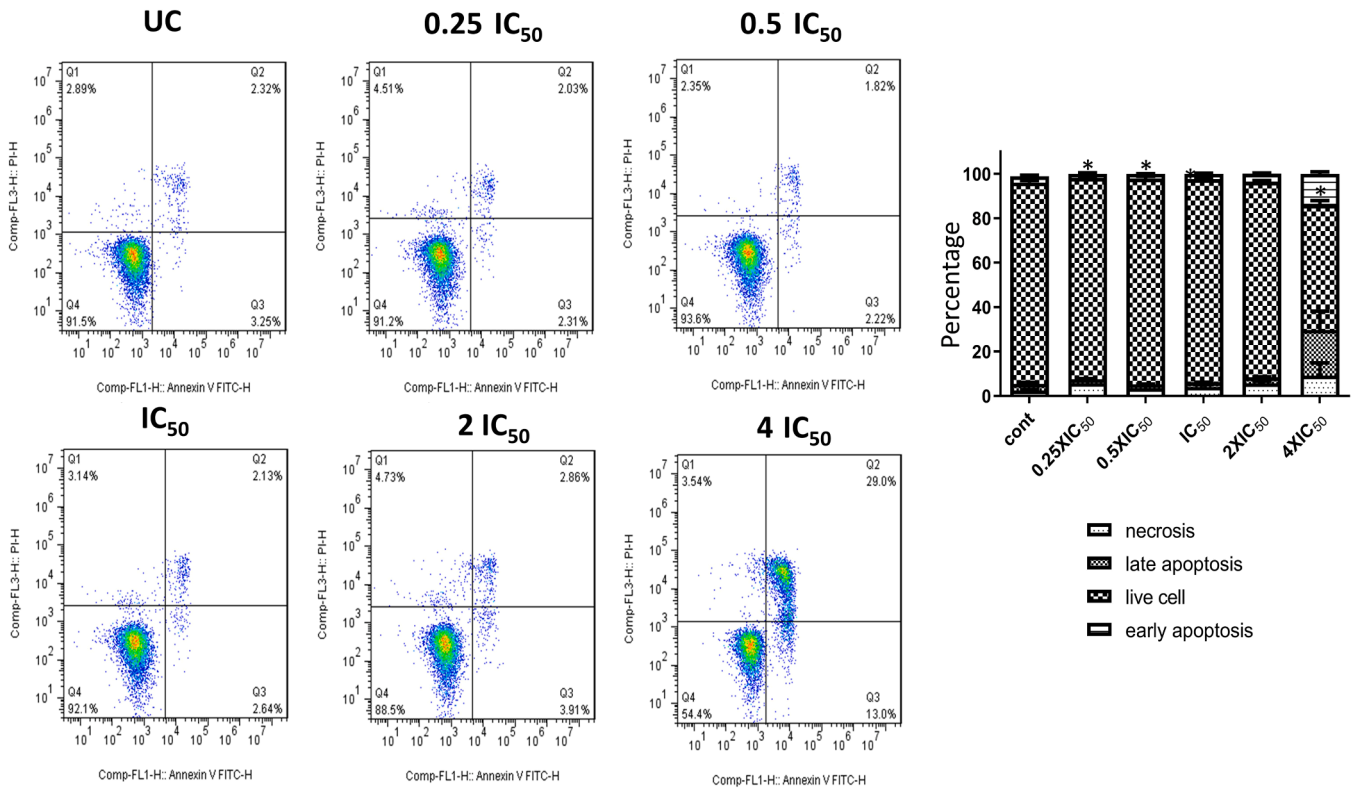


Fig. 8. Assessment of apoptosis by annexin V/PI staining in CCRF-CEM leukemia cells treated with different concentrations of candesalvone B methyl ester for 48h One representative experiment out of four performed ones is shown with numbers indicating the percentage of each population as compared to the control. Statistical analysis was done by paired student's *t*-test ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

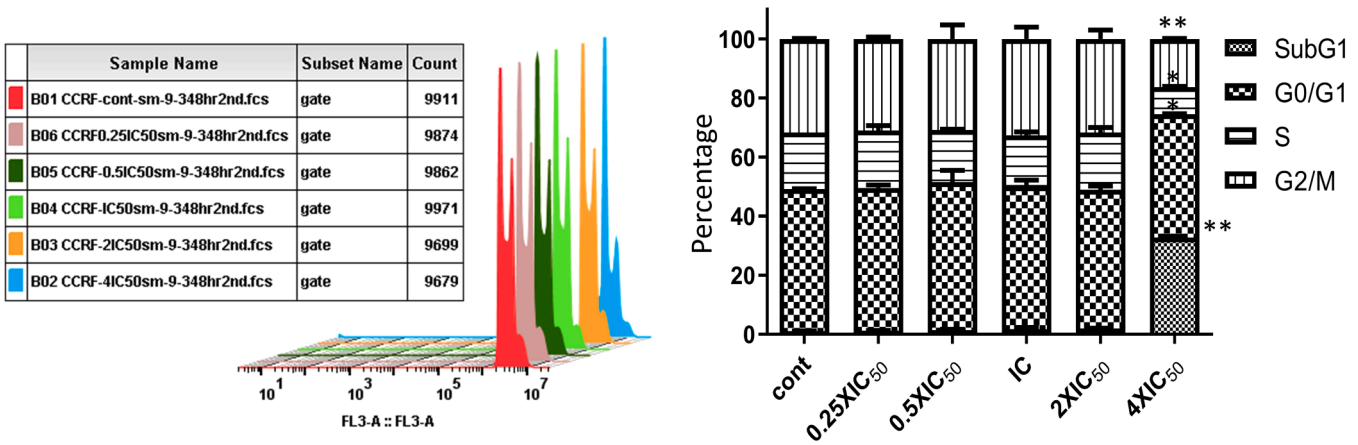


Fig. 9. Cell cycle analysis of CCRF-CEM cells upon treatment with various concentrations of candesalvone B methyl ester for 48h An increased accumulation of cells was observed in the sub-G1 phase, which was frequently associated with the concomitant induction of apoptosis due to the presence of DNA fragmentation. Statistical analysis was done by paired student's *t*-test ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

western blotting to be decreased in the CCRF-CEM cell line as a response to the increasing doses of candesalvone B methyl ester. RT-qPCR analysis validated the results of the western blot by downregulating the mRNA expression levels of *HSF1* in CCRF-CEM line. Additionally, the increasing concentrations of candesalvone B methyl ester were associated with increasing accumulation of cells in the sub-G1 phase of cell cycle up to 32.3 % indicating induction of apoptosis. The fraction of apoptotic cells rose to about 29.0 % after 48 h incubation with candesalvone B methyl ester in CCRF-CEM cells. Accordingly, candesalvone B methyl ester isolated from *S. multicaulis* Vahl. is a promising candidate that might be used alone or in combination with other anti-cancer agents to fight multidrug resistance in human leukemia.

Similarly, HSF1 inhibitors have been recently reported as novel anti-cancer agents. For instance, dorsomorphin reduced the phosphorylation of heat-stimulated HSF1, restrained its nuclear translocation, and rested the nuclear HSF1 levels in the cancerous cells, resulting in a reduction of HSPs expression (Li et al., 2019). Also, the dietary flavonoid fisetin induced programmed cell death in HCT-116 colon cancer cells via suppressing HSF1 activity, leading to prevention of HSP70 expression. However, due to a deficiency of fisetin cytotoxicity data, clinical trials with fisetin as an anti-cancer drug have not been initiated yet (Lall et al., 2016). Furthermore, Sharma and Seo, 2018, reviewed HSF1 inhibitors of natural origin including quercetin, stresgenin B, rocaglamide A, and CL-43. Moreover, schizandrin A, a direct natural inhibitor of HSF1

activity, which reduces the expression of HSPs, induced cell cycle arrest and apoptosis in colorectal cancer cells (Chen et al., 2020). Additionally, a terpenoid compound that was isolated from blister beetles and was used previously in Chinese medications, cantharidin, was reported to deactivate HSF1 (Naz et al., 2020). Another compound isolated from *Gastrodia elata*, 2, 4-bis(4-hydroxybenzyl)phenol, induced the degradation of HSF1 leading to cell cycle arrest and apoptosis (Vilaboa et al., 2017; Yoon et al., 2014). Vitexin indirectly inhibited the activity of HSF1, resulting in the reduction of proliferation and activation of colorectal cancer cells apoptosis (Bhardwaj et al., 2017). In parallel, IHSF115, a synthetic compound, also directly inhibited HSF1 and showed a wide range of cytotoxicity toward different human cancer cells including multiple myeloma, breast cancer, and Ewing sarcoma cell lines (Kijima et al., 2019). Additionally, Sharma and Seo (2018) reported other synthetic HSF1 inhibitors. The activity of another class of drugs depends on the targeting of HSPs (HSP inhibitors) instead of HSF1 to regulate the expression level and activity of HSPs (Mittal and Rajala, 2020).

Leukemic cells have the ability to re-program some of their metabolic pathways, particularly fatty acid metabolism which plays an important role in leukemogenesis, proliferation, signaling, metastasis, survival, energy production, and development of resistance to anticancer drugs (Germain et al., 2020; Koundouros and Poulogiannis, 2019; Castro et al., 2019). Thus, alteration in the fatty acid content of cells, especially by synthesis, and elongation, represents a hallmark of cancer and was associated with the poor prognosis of cancer patients (Tanaka et al., 2022). Elongase of very-long chain fatty acid 5 (ELOVL5) catalyzes the elongation of fatty acids which are important for the formation of phospholipids, and key cellular components of cell membranes to protect cells from oxidative stress and therapeutic drugs. ELOVL5 was upregulated in primary and resistant prostate cancer compared with nonmalignant prostate to promote tumor growth and metastasis. Membrane-bound transcription factors belonging to the SREBPs family, known as SREBF-1 and -2 control the expression of several genes encoding lipid synthesis enzymes. SREBP-1 preferentially activates ELOVL5 and ELOVL6 involved in the synthesis of fatty acids and triglycerides, whereas SREBP-2 preferentially activates proteins involved in the production of cholesterol and the LDL receptor (Moon et al., 2009). Thus, the knockdown of ELOVL5 in LNCaP and 22Rv1 human prostate cancer cells significantly attenuated the viability, colony formation, and proliferation which was associated with cell cycle arrest at the G1-S phase of both cell lines but did not induce death or apoptosis. Therefore, the therapeutic efficacy of ELOVL5 depletion is very significant and critical as a promising avenue for cancer treatment (Centenera et al., 2021).

Furthermore, ELOVL5 was elevated in prostate cancer cells, NE-like PCa, which are resistant to both androgen deprivation therapy and enzalutamide. This upregulation in the expression level of ELOVL5 was responsible for the development of drug resistance in prostate cancer. The mediation of drug resistance by ELOVL5 could be explained as follows: Upon elongation of fatty acids by the action of ELOVL5, the cell membrane content may alter with attached increasing of lipid rafts which are microdomains of plasma membrane containing high levels of cholesterol and sphingolipids and play the main role in cellular signaling transduction. As a result, AKT (protein kinase B)/mTOR (mammalian target of rapamycin) signaling will hyperactivate leading to enzalutamide resistance of prostate cancer cells (Xu et al., 2021). Accordingly, the targeting of ELOVL5 may facilitate the sensitivity of cancerous cells toward the therapeutic agents.

Candesalvone B methyl ester exerted anti-proliferation properties against P-glycoprotein-overexpressing CEM/ADR5000 leukemic cells in a dose-dependent manner. Besides, our microarray profiling and molecular docking studies along with the results of MST, western blot and RT-qPCR proved the regulation and targeting of ELOVL5 by candesalvone B methyl ester in a dose-dependent manner without significant effects on apoptosis or cell cycle distribution. For the variations between RT-qPCR, Western blotting, and microarray hybridization, the following points should be noted. Microarray analysis was performed at a single concentration (IC_{50}). In contrast, RT-qPCR and Western blotting analyses were conducted at three concentrations (IC_{50}, 2 × IC_{50}, and 4 × IC_{50}). At the IC*50* concentration, there were no significant changes in mRNA and protein expression levels. The microarray analysis at the IC_{50} dose showed a fold change of 1.45, indicating a relatively small upregulation of ELOVL5. However, at higher concentrations (2 × IC_{50} and 4 × IC_{50}), both RT-qPCR and Western blotting analyses showed coherent results, indicating downregulation. This inconsistency is not unexpected due to the different doses used, and it underscores the greater reliability of RT-qPCR and Western blotting at these concentrations.

Our outcomes are in agreement with the results of Centenera et al. (2021). Additionally, our data are in accordance with a study conducted by Nikulin et al. (2021), in MDA-MB-231 breast cancer cells, as they recorded no significant changes in the number of cells upon knockdown of ELOVL5 gene in both early and late stages of apoptosis. Moreover, referring to Xu et al. (2021), we conclude that targeting of ELOVL5 by candesalvone B methyl ester could diminish the acquired drug resistance through inactivation of the AKT/mTOR signaling pathway and increasing the anti-proliferative activity through downstream downregulation of P-glycoprotein (Zhang et al., 2020, 2019). AKT/mTOR activation was associated with poor prognosis, and strongly contributed to drug resistance in leukemia (Chen et al., 2021; Zhang et al., 2019, 2016; Evangelisti et al., 2013; Martelli et al., 2009). In contrast, Nikulin et al., 2021, concluded that according to the transcriptomic and proteomic analyses the knockdown of *ELOVL5* gene in MDA-MB-231 cells leads to an invasive-metastatic cascade of malignant breast tumors but with a weak activity.

Conclusion

In sum, we conclude that *S. multicaulisis* a natural plant enriched with diterpenes as promising candidates for the development of new anti-leukemic therapeutic agents. The bioactive compound isolated from the aerial parts of these plants, candesalvone B methyl ester, exerted cytotoxic mechanisms of action in leukemic cells including inhibition of proliferation, arresting of the cell cycle, inducing of apoptosis, and reducing the drug resistance mediated by P-glycoprotein. These actions were mediated through targeting of HSF1 and ELOVL5 signaling pathways in the sensitive human CCRF-CEM leukemia cell line and multidrug-resistant CEM/ADR5000 leukemic cells, respectively. *Salvia* plants may be an ideal safe choice for the development of potential therapies of leukemia in the future. Hence, more research is needed to explain the full complexity of pharmacological effects of the isolated phytochemical components of *S. multicaulis* by using in vitro and in vivo models, besides clinical trials.

CRediT authorship contribution statement

Heba K. Nabih: Writing – review & editing. **Rümeysa Yücer:** Writing – review & editing, Methodology. **Nuha Mahmoud:** Methodology. **Mona Dawood:** Methodology. **Mohamed Elbadawi:** Methodology. **Nasim Shahhamzehei:** Investigation, Validation. **Mohamed A.M. Atia:** Writing – review & editing, Methodology. **Ahmed AbdelSadik:** Writing – review & editing, Methodology. **Taha A. Hussien:** Methodology. **Mahmoud A.A. Ibrahim:** Writing – original draft, Methodology. **Sabine M. Klauck:** Writing – review & editing. **Mohamed-Elamir F. Hegazy:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Thomas Efferth:** Writing – original draft, Supervision.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.156023.

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