Promising Anti-leukemic Effect of Zataria Multiflora Extract in Combination with Doxorubicin to Combat Acute Lymphoblastic Leukemia Cells (Nalm-6)

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Research Article

Keywords: Zataria Multiflora, Doxorubicin, Acute Lymphoblastic Leukemia, Apoptosis, Combination therapy, Nalm-6 cells

Posted Date: August 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-778131/v1

Version of Record: A version of this preprint was published at Scientific Reports on July 25th, 2022. See the published version at https://doi.org/10.1038/s41598-022-16943-4.

Abstract

Purpose: One of the heterogeneous hematologic malignancies of the lymphocyte precursors or lymphoblasts is ALL. ALL has two incidence peaks that were determined in 2-5 years children and 60 years old adults. Cardiotoxicity of chemotherapeutic drugs is one of important side effect which may occur during or after chemotherapy period.

Methods: The aim of this study was to evaluate the effect of *Zataria Multiflora* extract (ZME), DOX, and ZME/DOX combination on Nalm-6 cells. In this vein, the cell viability was assessed by Trypan blue and MTT assay. Evaluation of apoptosis was also analyzed by Annexin-V/7-PI staining. Moreover, the expression of *Bax*, *Bcl-2*, *hTERT*, *c-Myc*, *P53,* and *P21* genes was detected by Real-Time PCR. Molecular docking as an in-silico method was performed for BCL2 and P53 as well.

Result: Our achievements indicated that ZME had dose-dependent effect on Nalm-6 cells and ZME synergistically potentiated DOX effect. The expression of *Bax*, *P53* and *P21* genes increased although the expression of *Bcl-2*, *hTERT*, and *c-Myc* genes decreased when cells treated with ZME/DOX combination. Molecular docking showed the interactions of Carvacrol and Thymol in the active cavities of BCL2 and P53.

Conclusions: Regarding to present study, ZME could be utilized as a combinatorial and potential drug for leukemic patients, which is under the treatment by DOX due to reducing the chemotherapy drug doses.

1. Introduction

Acute Lymphoblastic Leukemia (ALL) is a hematologic malignant disorder originating from either T- or B-cells lymphoid precursors. B-cell accounts for 80–85% of ALL cases, even as T-ALL accounts for 15–20% of ALL cases. Among childhood cancer ALL is the most common and the third common leukemia through adults. Whereas ALL in children is more common than ALL in adults, childhood ALL has desirable prognosis and outcomes [1, 2]. The complete remission rates are 85 to 90% and long-term survival rates are 30 to 50% through using intensive conventional chemotherapy regimen. ALL may have several relapses and may lead to dead [3, 4]. Treatment among relapsed pediatric patients due to high toxicity regimen and low remission rates is not promising in second and subsequent relapses in following years of diagnosis [5]. Such an effective anti-leukemic potential and lower toxicity alternatives are necessary for these pretreated patients [6].

Doxorubicin-associated cardiotoxicity can occur during or after therapy and it leads to asymptomatic left ventricular (LV) dysfunction, cardiomyopathy, heart failure, and, in some cases cardiac death [7, 8]. Despite researchers tried to improve strategies of leukemia treatment, their efforts have not yet achieved favorable outcomes. Relapse of the disease is still a major problem in ALL patients. Doxorubicin (DOX) from anthracyclin-antibiotic family is one of the most effective anti-tumor drugs, which has an important role in the first-line of ALL treatments [9]. Even though most of the patients with childhood leukemia are treated with DOX and they survive a long time after therapy. Many of them encounter a wide range of side

effects such as cardiomyopathy and congestive heart failure, that it increased risk of sudden death [10]. In order to decrease the toxicity of DOX, physicians use limited dose and combined-modality strategies [11, 12].

In the last decades, various drugs are applied to treat the malignancies but some of them have a lot of undesirable effects and most of them are not efficient enough [13]. Accordingly, searching to find new drugs with fewer side effects is one of the most important aims in cancer therapies. Nowadays, plant extracts and natural products are the best choice in cancer therapy due to their fewer side effects [14]. One of the members of the Lamiaceae family is *Zataria multiflora* (ZM) that is found in warm areas of Iran, Pakistan, and Afghanistan. ZM is used as spices to give foods odor and flavor [15]. In addition to its traditional applications, some studies indicate anti-spasmodic, antiseptic, and anesthetic effect of ZM extract (ZME). According to recent studies, researchers have demonstrated that ZME has various effects such as analgesic, anti-inflammatory, anti-nociceptive, anti-microbial and anti-oxidant properties [16–19]. Furthermore, its anti-tumor effect of ZME was investigated on some cancer cell lines [20]. In this study we assessed the apoptotic effect of ZME, DOX, and ZME/DOX combination on NALM-6 cells. ZME may potentiate the apoptotic effect of DOX in order to decrease its side effects.

2. Results

ZME and DOX induce cell death on pre-B ALL cells

The viability percent of Nalm-6 cells treating with various concentrations of ZME and DOX individually and in combination were evaluated using trypan blue assay at 24, 48, 72 h after treatment (Fig 1). The results demonstrated that the ZME and DOX decreased the viability of Nalm-6 cells. DOX had a dose- and time-dependent effect on cells, although ZME had only a dose-dependent effect. A significant decrease in viability of Nalm-6 cells was also seen in combination treatment. Here, interactions of the combination drug were evaluated using isobologram and combination index (CI) analyses. The isobologram analyses were conducted to assess the synergic effect of ZME and DOX on Nalm-6 cells. The CI-Fa curve (Fig 2) indicated the synergistic effects (CI < 1) of all combination doses. As represented in Fig 2, isobologram analysis indicated that all the points are below the line of additive effects in the synergism area. Moreover, the fraction-affect (FA) versus combination index analysis also demonstrated the synergistic (CI < 1) anti-proliferative effect of ZME/DOX combination on Nalm-6 cells (Table. 2).

ZME potentiated the cytotoxicity effect of DOX

Metabolic activity of treated cells with ZME and DOX was investigated with the MTT colorimetric method. As shown in Fig. 3 (a, b), ZME and DOX separately had cytotoxicity effect on Nalm-6 cells and they reduced the metabolic activity of treated Nalm-6 cells. Fig. 3 (c) presents the results of combination doses of ZME and DOX. As seen, they were more efficient than individual doses. Outstandingly, the

cytotoxic effect of DOX on Nalm-6 cells was potentiated by ZME using the synergistic combination treatments. Furthermore, two concentrations of DMSO did not have significant effect on Nalm-6 cells.

ZME enhanced the effect of DOX on programmed cell death

Here, 100 µg/mL ZME plus 10nM DOX as a combination dose besides 100µg/mL ZME and 10nM DOX as individual doses were selected for investigation of Nalm-6 cells apoptosis. As shown in Fig. 4, the combination dose had a considerable increase in the percentage of Annexin-V and Annexin-V/PI positive cells. Consequently, a combination of 100 µg/mL ZME and 10nM DOX induced 43.4% apoptosis, which is more than 23.71% apoptosis caused by 10 nM DOX alone ($P < 0.0001$).

Inductive Effect of ZME/DOX Combination on The Gene Expression

Nalm-6 cells were exposed to 100 µg/mL ZME, 10nM DOX and a combination of them for 48h. Thereupon, the expression of target genes was evaluated using quantitative real-time PCR. According to the results shown in Fig. 5, the expression of the *Bax* gene increased in the combination dose more than the single doses. These results also indicated the significant decreasing rate in *Bcl-2* gene expression which was influenced by the combination dose. Furthermore, *Bax* and *Bcl-2* expression ratio presented that the ZME enhances the DOX apoptotic effect on Nalm-6 cells (Fig. 5).

P53 and *P21* were chosen as tumor suppressor genes to assess them in Nalm-6 cells treated with 100 µg/mL ZME, 10nM DOX and combination of them after 48h. As presented in Fig. 6 (a, b), the expression of the *P53* and *P21* genes was increased individually and combination dose of ZME/DOX increased the gene expression more than single doses of ZME and DOX.

As the results are shown in Fig. 6 (c, d), 100 µg/mL ZME, 10nM DOX and a combination of them led to reduce the expression of *hTERT* and *c-Myc* genes. The combination dose of ZME/DOX had more effective than ZME and DOX single doses.

ZME had No Significant Effect on PBMCs

PBMCs were selected as a human normal cell to evaluate the effect of various concentrations of ZME. This study assessed the effect of ZME on PBMCs using MTT and flowcytometry assays in 100 µg/mL and 200 µg/mL concentrations after 48h treatment. The results demonstrated that ZME had no significant impact on PBMCs.

Molecular Docking

The ligand binding residues of *BCL2* and *P53* were identified by CASTp server. According to CASTp ligand binding site of *BCL2* in chain B includes: Lys22, Gly25, Arg26, Asp102, Phe104, Ser105, Tyr108, Arg109, Glu114, Met115, Ser116, Glu152, Val156, Val159, Glu160, Asn163 and also the *P53* active site for ligand binding includes: Phe55, Gly58, Gln59, Met62 (chain A), Trp23, Lys24 (chain B). Due to figure out the interaction among the structures and activities of our cytotoxic agents, molecular docking has been conducted under similar conditions via the AutoDock 4.2 tools program. According to our result, thymol and carvacrol as ligands bind to the residues of *P53* and *BCL2* cavities with various bond and energy binding (Table.3, 4 And Fig. 7). These interactions enhance the apoptosis pathway on Nalm-6 cells treatment with ZME and they may confirm the apoptotic effect of ZME on Nalm-6 cells.

3. Discussion

With the increase of cancer incidence, many researches have been focused on new chemotherapeutic treatments [27]. One of the most useful and effective chemotherapeutic drugs is DOX which is used to treat wide groups of cancers, such as hematologic malignancies, leukemias, lymphomas and solid tumors like breast cancer. Todays, DOX is utilized widely in the chemotherapeutic regimens of cancer patients, even though DOX has a lot of side effects on patients [28, 29]. Cardiotoxicity is one of the most important side effects of DOX causing cardiomyopathy, arrhythmia, congestive heart failure and finally heart failure in patients during or after treatment. To decrease the side effects, the dose of DOX should be reduced [30, 31]. Nowadays, the cytotoxic effects of plant extracts on malignant cells are considered to use them as an alternative or complementary drug in chemotherapeutic treatment [32]. The plant extracts have low complications and they can combine with the low doses of the chemotherapeutic drug in treatments to enhance the effect of drugs [33]. The results of this study indicated the anti-tumoral effect of ZME individual and in combination with DOX on Nalm-6 cell line. The present study also evaluated the apoptotic effect of ZME and DOX individually and in combination on Nalm-6 cells. The present study indicated the notable synergistic apoptotic effect of ZME and DOX on the Nalm-6 cells. As shown in our results, the viability of Nalm-6 cells was decreased in exposure to ZME and DOX and their viability in combination was decreased more than individual doses. Furthermore, doses of ZME accompanied by DOX concentrations potentiated the cytotoxicity effect on Nalm-6. In this regard, Janitermi el al. according to their MTT results, claimed that ZME had time- and dose-dependent cytotoxic effect on some cancer cell lines (MCF-7, AGS & HeLa) and it decreased the viability and metabolic activity of treated cancer cells. They also reported that ZME had no cytotoxicity effect on fibroblast cells as a normal cell [34–36]. In this way, our results from flowcytometry and MTT indicated that there was no considerable cytotoxicity effect on PBMCs that were exposed to 100 and 200 µg/mL ZME. Nevertheless, some previous studies investigated the apoptotic effect of ZME components like Thymol, Carvacrol, and P-Cymen on malignant cell lines. Yi Li and colleagues reported that Thymol downregulated the *Bcl-2* and *Bcl-Xl* expression while it upregulated the *P21* expression in bladder cancer cell lines. They also showed that Thymol did not have a cytotoxic effect on the urothelial cell line as non-malignant cell [37]. Flowcytometry and gene expression results illustrated that ZME provoked the apoptotic effect of DOX on NALM-6 cells. The expression of *BAX* as we expected, has increased on treated cells with ZME and DOX.

The *Bcl-2* expression was down-regulated on exposed cells with ZME and DOX. Moreover, the expression of c-Myc and *hTERT* had been reduced greater in combined with ZME and DOX. In another experiment, Punia et al. also indicated that a combination of DOX and Acacetin (a plant derivative) enhanced the apoptotic effect on one type of lung carcinoma cells [38]. The increasing of *BCL2* as an anti-apoptotic factor in many cancers could be a promising target to combat malignant cells. Since *BCL2* inhibitors could occupy BH2 and BH3 positions and on the other hand according to our molecular docking analysis, carvacrol and thymol could interact with *BCL2* in BH3 and BH1 positions, they may inhibit *BCL2* to lead cancer cells in the apoptosis pathway [39]. Therefore, these studies show the plant derivatives beside the alternative drugs and methods such using of nanoparticles, can act as drug complement in clinical therapies, particularly chemotherapeutic treatments [40]. In addition to mentioned, our molecular docking analysis demonstrated that ZM components matched the molecules involving in apoptosis and they may trigger the apoptosis pathway. Severe side effects are the most problem of chemotherapy drug. Due to attain a solution to eliminating the problem, researchers attempt to achieve alternatives or complements. Subsequently, the investigation of plant extracts on malignant cells is one of the best elections to induce the programmed cell death. As illustrated in the present study, ZME may be utilized as an alternative or a complement to decrease the dose of DOX for pre-B acute lymphoblastic leukemia cells.

4. Material & Methods

Ethics statements

This study was approved by Kerman University of Medical Science Ethical & Research Committee (ethical code: IR.KMU.REC.1398.397). The volunteers were informed about the objective and procedure of the study and those who were willing to participate, donate blood sample and sign an informed consent were recruited. All methods were performed in accordance with relevant guidelines and applicable regulations.

Cell culture

Acute Lymphoblastic Leukemia cell line (Nalm-6, NCBI C212) was obtained from the Pasteur Institute collection, Tehran, Iran. Nalm-6 cells were cultured in RPMI-1640 with 2mM L-glutamine (Gibco™ A1049101) containing 10% fetal bovine serum (FBS) (Gibco™ A3160402), 1% antibiotic (Penicillin–Streptomycin Solution 100X, Biowest, L0022) in a humidified atmosphere of 5% CO_2 incubator at 37 °C. ZME was prepared according to methanolic extract protocol that Saedi Dezaki et al. applicated in their study [21]. The stock of DOX (EBEWE Pharma, Austria) was also diluted into considered concentration for treatment.

Trypan Blue assay

To evaluate the apoptotic effects of ZME and DOX on cell viability, Nalm-6 cells (250×10^5 cells/mL) were seeded in 12-well plate and incubated in the presence of the various concentrations of ZME (20, 40, 80, 100, 200 µg/mL) and DOX (5, 10, 20, 40, 80,100 nM) individually for 24, 48, and 72 h. After that, the cell suspension was centrifuged and the cell pellet was suspended in a serum-free complete medium. Next, one part of 0.4 % trypan blue (Gibco™ 15250061) and one part of cell suspension was mixed and then allowed mixture to incubate 2 minutes at room temperature. The total number of unstained (viable) and stained (non-viable) cells was manually counted by Neubauer chamber and light microscope (ECLIPSE E100, Nikon). Finally, the percentage of viable cells was calculated as “Viability (%) = viable cells / viable cells + death cells ×100” [22].

Determination of Combination Index and Dose Reduction Index

To estimate the interaction between ZME and DOX, the combination index (CI) was calculated using CompuSyn Software (ComboSyn, Inc., Paramus, NJ, USA) according to the classic isobologram equation: “$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$”, where $(Dx)_1$ and $(Dx)_2$ represent the individual dose of ZME and DOX required to inhibit a given level of viability index, and $(D)_1$ and $(D)_2$ are the doses of ZME and DOX necessary to produce the same effect in combination, respectively. Since different CI values (<1, =1, >1 indicate synergism, additive effect, and antagonism, respectively) can be observed at different levels of growth inhibition (fraction affected, FA), CI versus FA plots were applied to present the data using MS Excel. The dose which may be decreased in a combination for a given level of effect as compared to the concentration of individual drug alone defined as dose reduction index (DRI) and calculated as follow: $(DRI)_1 = (Dx)_1/(D)_1$ and $(DRI)_2 = (Dx)_2/(D)_2$ [23, 24].

MTT assay

In vitro screening of the cytotoxicity effect of ZME and DOX towards cancer cell lines was measured using MTT colorimetric assay. The metabolization of thiazolyl blue tetrazolium bromide into formazan crystals by Nalm-6 alive cells was assessed by this test [25]. Hence, 1×10^4 Nalm-6 cells were seeded in 96-well plates with various concentrations of ZME (20, 40, 80, 100, 200 µg/mL) and DOX (5, 10, 20, 40, 80,100 nM) individually for 24, 48, and 72 h. Afterward, the plate was centrifuged at 700×g for 10 min and the supernatant was removed. The cells were incubated with 100 µL MTT solution (0.5 mg/mL; (M5655, Sigma) at 37 °C. After 4 h, the formazan crystals were solubilized by the addition of 150 µL dimethylsulfoxide (DMSO) (Merck, CAS 67-68-5) at each well and optical absorbance was evaluated at 570 nm with an enzyme-linked immunosorbent assay reader. The percentage of metabolic activity of treated cells was calculated relative to untreated cells which were set as negative control. In addition, MTT test was performed for combination dose of ZME/DOX (100 µg/mL ZME +10 nM DOX, 100 µg/mL ZME +20 nM DOX, 200 µg/mL ZME +10 nM DOX, 200 µg/mL ZME +20 nM DOX). In addition to untreated

cells, NALM-6 were treated with the highest concentrations of DMSO (were used in our study) as a negative control owing to use it for dissolving ZME (0.01% and 0.1%).

Flowcytometry

The flowcytometry technique was used to assess the effect of ZME and DOX on the induction of early and late apoptosis using annexin V-propidium iodide (PI) staining [26]. Consequently, 4×10^5 NALM-6 cells were seeded into six-well cell culture plates. Then, after 48 h, the cells were collected and they were washed with PBS. Flowcytometry was performed using Annexin-V Apoptosis Detection Kit (Mab Tag, AnxF100PI) and the results were analyzed using the FlowJo.7.6.1 software.

RNA isolation and preparation of cDNA

YTzol Pure RNA (Yekta Tajhiz Azma, YT9066) was used to isolate total RNA from untreated (control) and treated cells with 100 µg/mL ZME, 10 nM DOX and ZME/DOX combination (100 µg/mL ZME and 10 nM DOX). Quantity of RNA samples was assessed by NanoDrop (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE) at A260/A280 ratio. The quality and purity of extracted RNA were illustrated by agarose gel electrophoresis. Reverse transcription (RT) reaction was carried out according to the manufacturer instructions using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific Fermentas, K1622).

Quantitative Real-time PCR

Changes in mRNA expression of desired genes were surveyed by real-time PCR. Quantitative real-time PCR was performed by 10 µL containing Real Q Plus 2x Master Mix Green (Amplicon, Denmark, A325402), 1.5 µL of the cDNA product, 1 µL of forward and reverse primers (10 pmol of each other), and 7.5 µL of nuclease-free water. Thermal cycling conditions included an initial activation step at 95 °C for 15 min followed by 40 cycles, a denaturation step at 95 °C for 15s and a combined annealing/elongation step at 60 °C for 60s. The reaction took place in the RotorGene® Q Real-time PCR System (Qiagen, USA). A melting curve analysis was performed to verify the specificity of the products. The fold change was measured relative to the control and calculated after adjusting for the B-actin reference gene using Ct ($2^{-\Delta\Delta CT}$) method. Nucleotide sequences of the primers used for real-time RT-PCR listed in Table. 1.

ZME Effect on Normal Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor using density gradient centrifugation using Ficoll- Hypaque density gradient (Lymphodex, Germany). Isolated cells were washed two times by PBS. Thus, the pellet was resuspended in 1 mL complete media (containing RPMI-1640 with 2mM L-glutamine, 10% FBS and 1% antibiotic) and cultured in T75 flasks with 8 mL complete media at

the same condition used for Nalm-6 cells. These cells were treated with 100 and 200 µg/mL ZME and incubated at 37 °C. Then, metabolic activity and apoptotic treated cells were assessed using MTT and flowcytometry after 48h, respectively.

Molecular Docking

The crystal structure of *P53* (PDB ID: 1TUP), *BCL2* (PDB ID: 2XA0) was obtained from protein data bank (https://www.rcsb.org). In addition, the DNA chain (1TUP) and BH3 (2XA0) peptide were eliminated by using the software MOE 2019.102 (Molecular Operating Environment). Thymol (CID:6989), carvacrol (CID:10364) structures have also been acquired from PubChem compound database. CASTp (Computed Atlas of Surface Topography of proteins) server was used to predict the *P53* and *BCL2* pocket and cavities of active sites for ligands binding. CASTp identifies not only the cavities but also pockets and pocket mouth opening. Here, AutoDock Tools 4.2 has been used for molecular docking. The grid box dimension has been adjusted to 126x126x126 Å surrounding the active site of the protein in order to ensure the free rotation of the ligands in the inner side of the grid. The docking run numbers have been estimated to be 100. The resulting poses have been chosen according to the corresponding binding energy. By using protein-ligand interaction profiler server, the interactions of ligand and residues of protein were investigated. Ultimately, the 3D shapes of the final interaction were drawn by using the software Chimera 1.12.

Statistical Analysis

Experimental data are expressed by mean ± standard deviation (SD) to compare the mean values among experimental groups using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). All tests were done in duplicate or triplicate. Statistical analysis of MTT and Trypan blue data was calculated by Two-way ANOVA test and One-way ANOVA analysis was used to evaluate the data of flowcytometry and Quantitative real-time PCR. Statistically different values were defined significant at $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

Declarations

Acknowledgments

We would like to be grateful to paramedical faculty of Kerman university of medical sciences for supporting this study by grant No. 96000991.

CRediT authorship contribution statement

Mahla Lashkari: Investigation, Software, Formal analysis, Methodology, Writing- Original Draft and Writing- Review & Editing. Ahmad Fatemi: Methodology, Formal analysis and Project administration. Hajar Mardani Valandani: Methodology, Project administration. Roohollah Mirzaee Khalilabadi: Conceptualization, Methodology, Data Curation, Supervision, Funding acquisition and Writing- Review & Editing.

Funding:

This work was supported by Kerman University of Medical Sciences [No. 96000991].

Conflicts of interest/Competing interests:

The authors declare that they have no competing interest.

Ethical approval:

This study was confirmed by Kerman University of Medical Sciences Research Committee; ethical code: IR.KMU.REC.1398.397.

Author agreement:

All authors have seen and approved the final version of the manuscript.

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Tables

Table 1

Primer sequences used for quantitative real-time PCR.

Gene	Forward primer	Reverse primer
B-actin	5’-CCAACCGCGAGAAGATGA-3’	5’-TCCATCACGATGCCAGTG-3’
hTERT	5'- CGGAAGAGTGTCTGGAGCAA -3'	5'- GGATGAAGCGGAGTCTGGA -3'
c-MYC	5'-GTCCTCGGATTCTCTGCTCTC-3'	5'-CAACATCGATTTCTTCCTCATCTTC-3'
P21	5'-CCTGTCACTGTCTTGTACCCT-3'	GCGTTTGGAGTGGTAGAAATCT-3'
P53	5'-CTGGCCCCTGTCATCTTCTG-3'	5'-CCGTCATGTGCTGTGACTGC-3'
Bax	5’-AGGATCGAGCAGGGCGAATG-3’	5’-TCAGCTTCTTGGTGGACGCA-3’
Bcl-2	5’-ATCGCCCTGTGGATGACTGAG-3’	5’-CAGCCAGGAGAAATCAAACAGAG-3’
Bcl-xl	5’-TGCATTGTTCCCATAGAGTTCCA-3’	5’-CCTGAATGACCACCTAGAGCCTT-3’

Table 2

CI and DRI for drug combination by ZME and DOX

ZM (µg/ml)	DRI	Doxorubicin (nM)	DRI	CI value (At inhibition of 50%)
100	3.256	10	4.960	0.508
200	1.925	10	5.653	0.696
100	4.666	20	3.284	0.518
200	1.547	20	2.377	1.069

Table 3

The best binding energy and RMSD from docking molecular analysis.

	Binding Energy (kCal/mol)	**RMSD** (Å)
BCL2 / Carvacrol	-5.59	0.053
BCL2 / Thymol	-5.45	0.191
P53 / Carvacrol	-5.85	0.011
P53 / Thymol	-5.69	0.041

Table 4

The hydrophobic interactions and hydrogen bonds of BCL2 and P53 residues with Carvacrol and Thymol.

		Carvacrol	**Thymol**
BCL2	**Hydrophobic Interactions**	ARG109, GLU114, VAL156, VAL159	ARG109, GLU114, MET115, VAL156, VAL159
	Hydrogen Bonds	SER116	SER105, GLU152
P53	**Hydrophobic Interactions**	LYS24, GLU28, LEU54, PHE55	LYS24, GLU28, LEU54, PHE55
	Hydrogen Bonds	TRP23, LEU26	LYS51

Figures

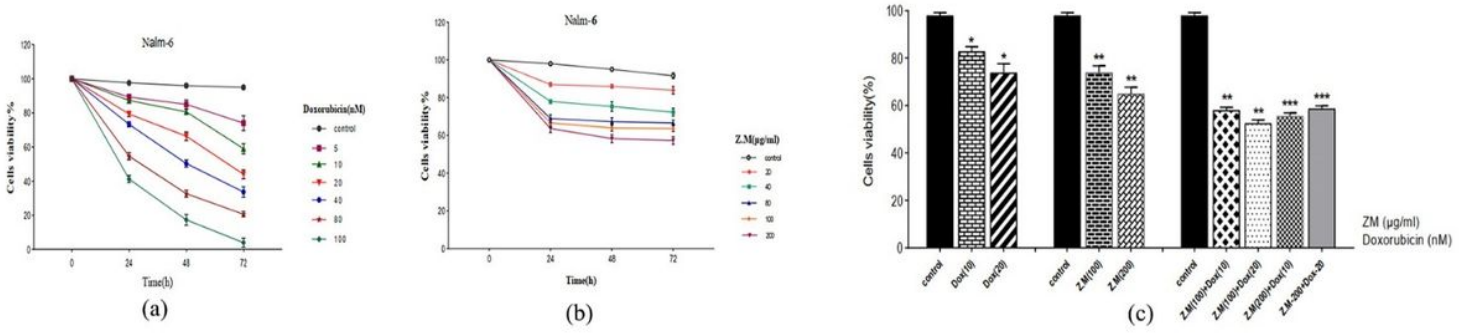

Figure 1

The ZME (a) and DOX (b) effect of various doses on the viability of Nalm-6 cells using trypan blue assay (mean ± SE, n = 3). These graphs present the changes of ZME and DOX apoptotic effect after 24, 48, and 72 h. As shown, different concentrations of ZME and DOX had dose-dependent effect, dose and time-dependent effect, respectively. Effect of ZME and DOX combination and single doses (c) after 48h on the viability of Nalm-6 cells using trypan blue exclusion assay (mean ± SE, n = 3). As shown, combination doses had a more significant apoptotic effect than individual doses on Nalm-6 cells (*P < 0.05, **P < 0.01, ***P < 0.001, relative to untreated cells).

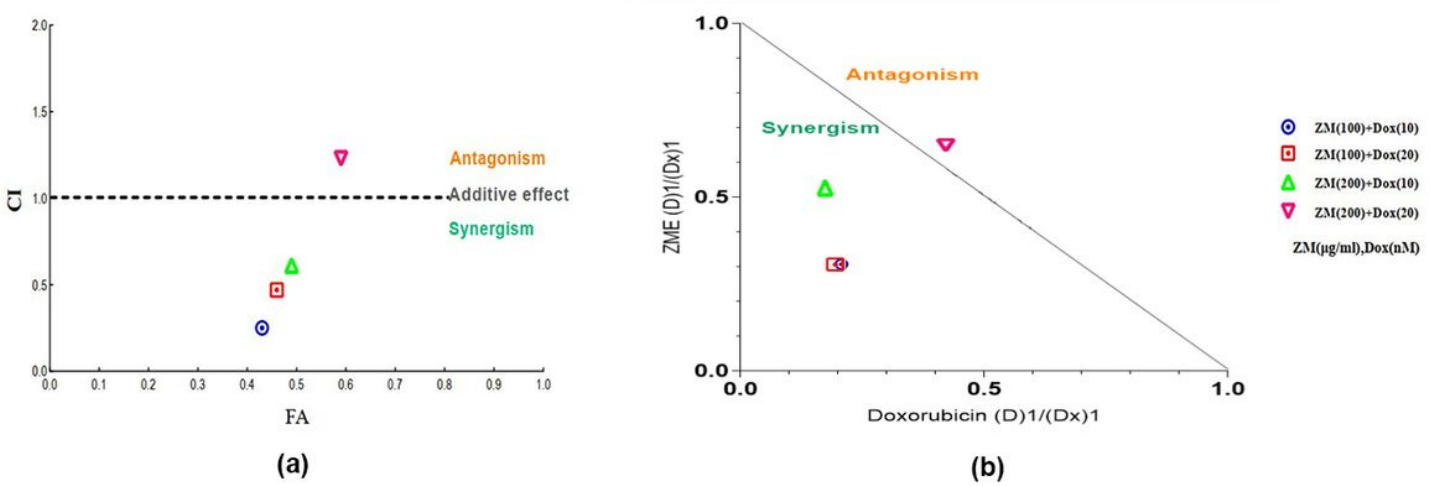

Figure 2

The combination index (CI) versus fraction effect (FA) curve (a) of ZME and DOX combination treatment. Exposure of Nalm-6 cells with various combinations of ZME and DOX and cell viability values of trypan blue assay was used for CI vs. As reported by FA curve, the CI < 1, =1, and > 1 indicate respectively synergism, additive effect (solid line), and antagonism effect. Combinations of 100 μg/mL ZME+10 nM DOX on Nalm-6 cells demonstrated the most desirable synergism effect among other combinations. Dose-normalized isobologram analysis of ZME and DOX combination. The CI was calculated according to the normalized isobologram equation (b). (Dx)1 and (Dx)2 indicate the individual dose of ZME and DOX required to inhibit a given level of viability index, and (D)1 and (D)2 are the doses of ZME and DOX necessary to produce the same effect in combination, respectively. Antagonism effect is represented by above points of the effect line, whereas the points are below the effect line demonstrate the synergism effect. Three combination points for Nalm-6 cells were below the effect line, so showed synergism effect.

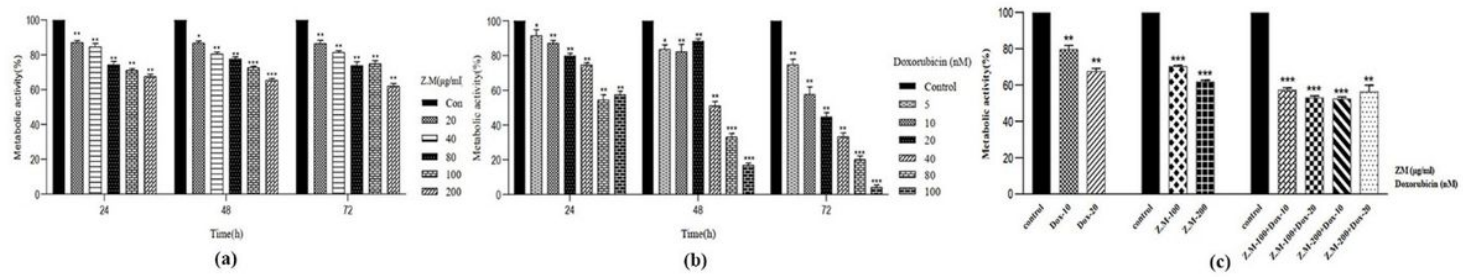

Figure 3

The effect of ZME (a) and DOX (b) on the metabolic activity of Nalm-6 cells 24, 48, 72 h after treatments. As shown, ZME had dose-dependent effect but DOX not only had dose-dependent effect but also had time-dependent effect. The effect of ZME and DOX combination against single doses after 48h treatment on metabolic activity of Nalm-6 cells (c). The significant effect of drugs combination is visible in four doses (ZME and DOX: 100+10, 100+20, 200+10, 200+20) and we use ZME: 100 µg/mL and DOX:10 nM as a combination dose for our study (*P < 0.05, **P < 0.01, ***P < 0.001, relative to untreated cells).

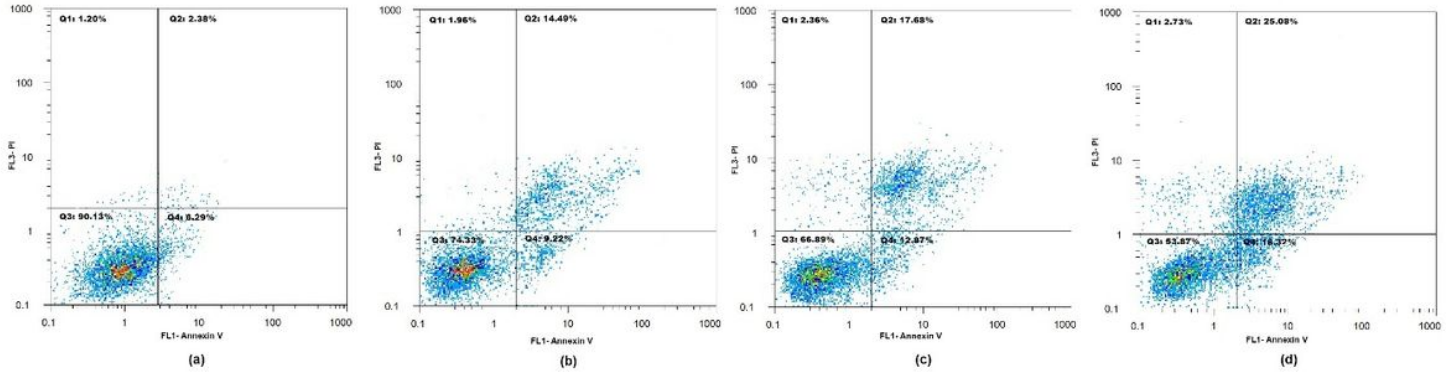

Figure 4

Nalm-6 cells were treated in a selected combination and individual: control (untreated) (a), DOX: 10 nM (b), ZME:100 µg/mL (C), ZME: 100/DOX: 10 (d) for 48h. Then, cells were analyzed for Annexin-V and Annexin-V plus Propidium Iodide (PI) by flow cytometry. As shown, ZME significantly enhanced the apoptotic effect of DOX on Nalm-6 cells in combination with more than an individual after 48h treatment.

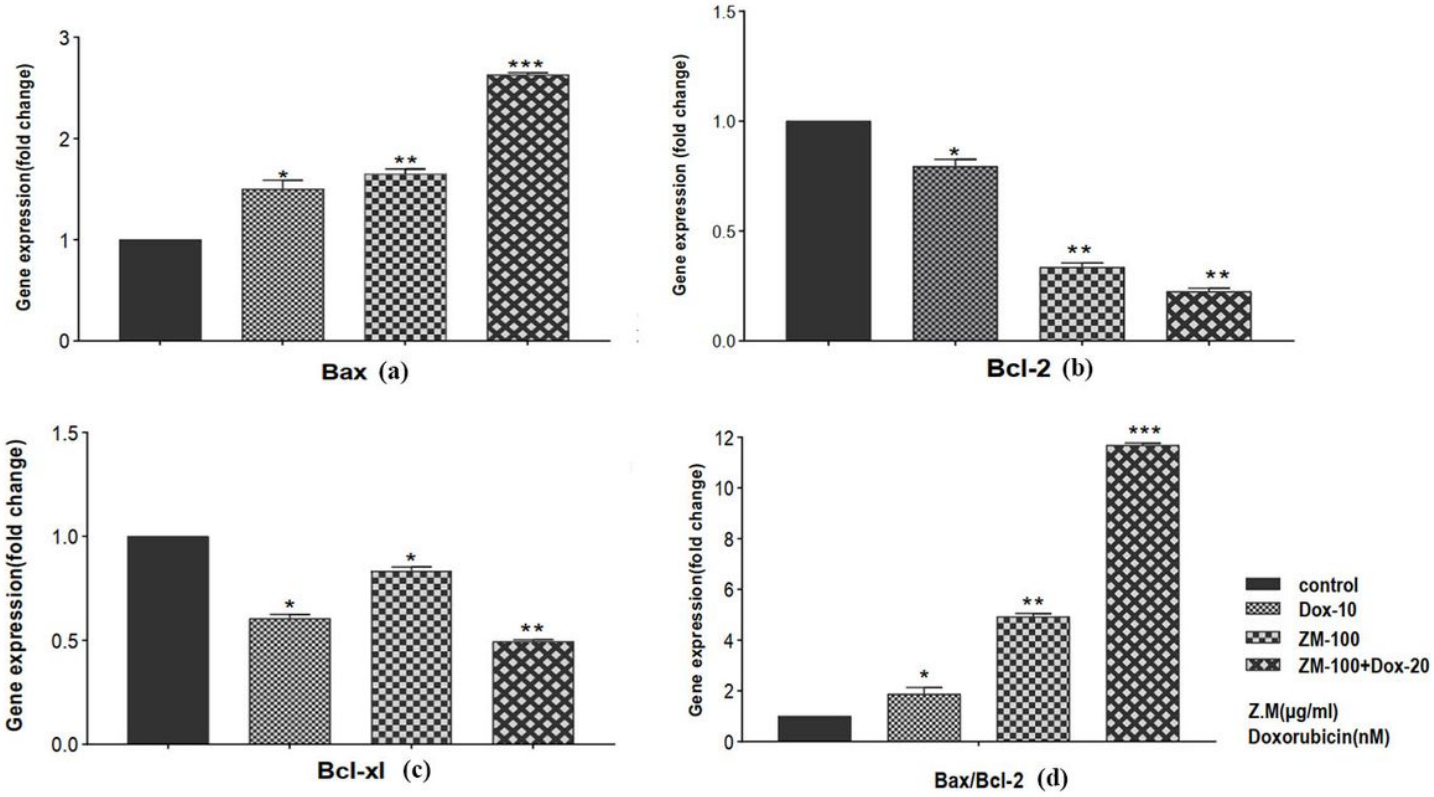

Figure 5

Fold change gene expression. Graph presents that ZME and DOX upregulate the Bax gene (pro-apoptotic) and down-regulates Bcl-2 gene (anti-apoptotic). A combination dose of ZME and DOX changes gene expression more than single doses on Nalm-6 cells after 48h treatment. Bax and Bcl-2 ratio also was shown in this figure and demonstrated the significant effect of ZME and DOX in combination dose. In addition, Bcl-Xl from Bcl-2 family was upregulated by ZME and DOX treatment in Nalm-6 cells after 48h (*P < 0.05, **P < 0.01, ***P < 0.001, relative to untreated cells)

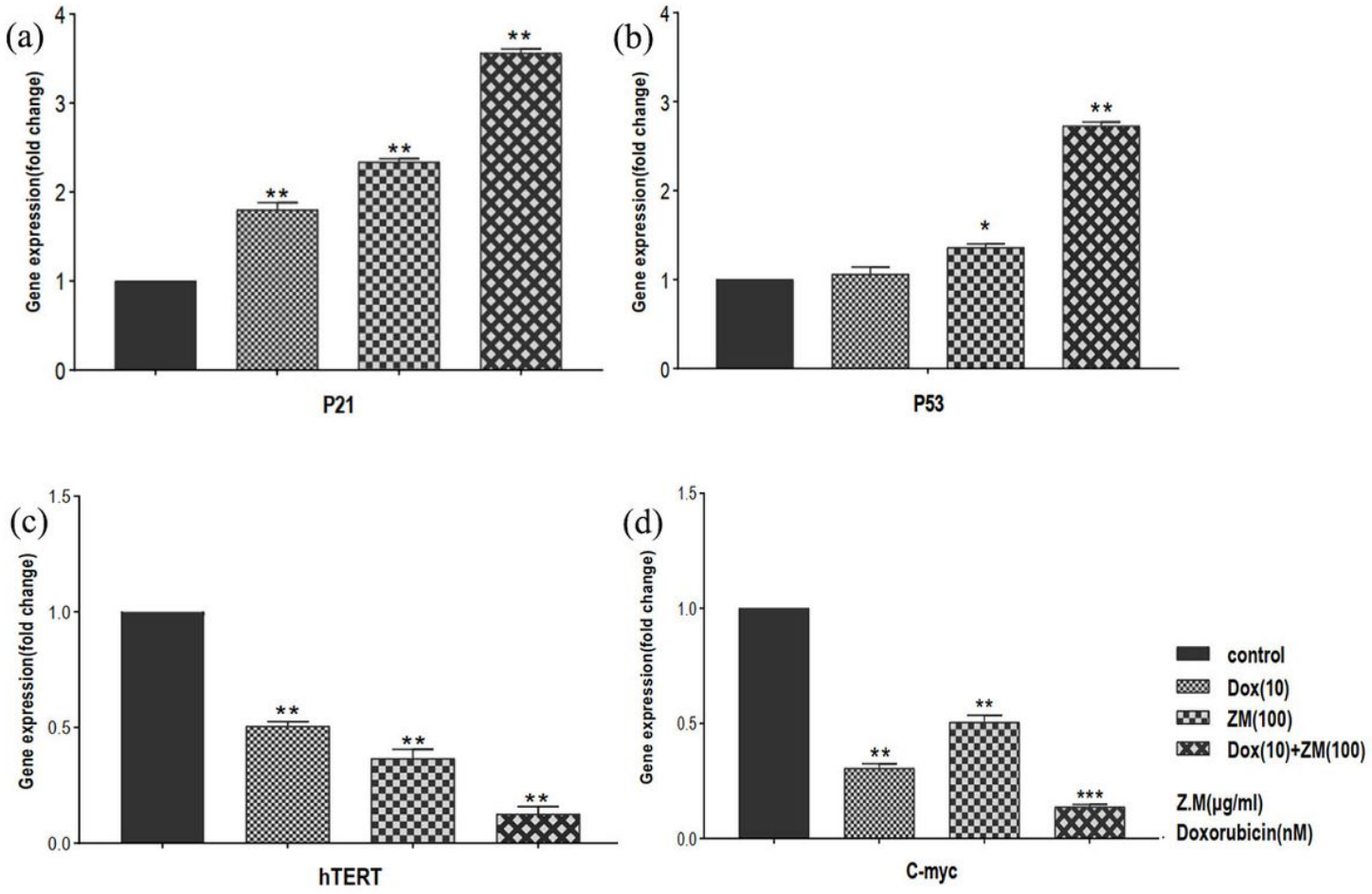

Figure 6

Gene expression graph presents that ZME and DOX down-regulates the h-TERT gene and c-Myc gene. A combination dose of ZME and DOX changes gene expression more than single doses on Nalm-6 cells after 48h treatment. Fold change gene expression graph presents that ZME and DOX up-regulate P21 gene and P53 gene.

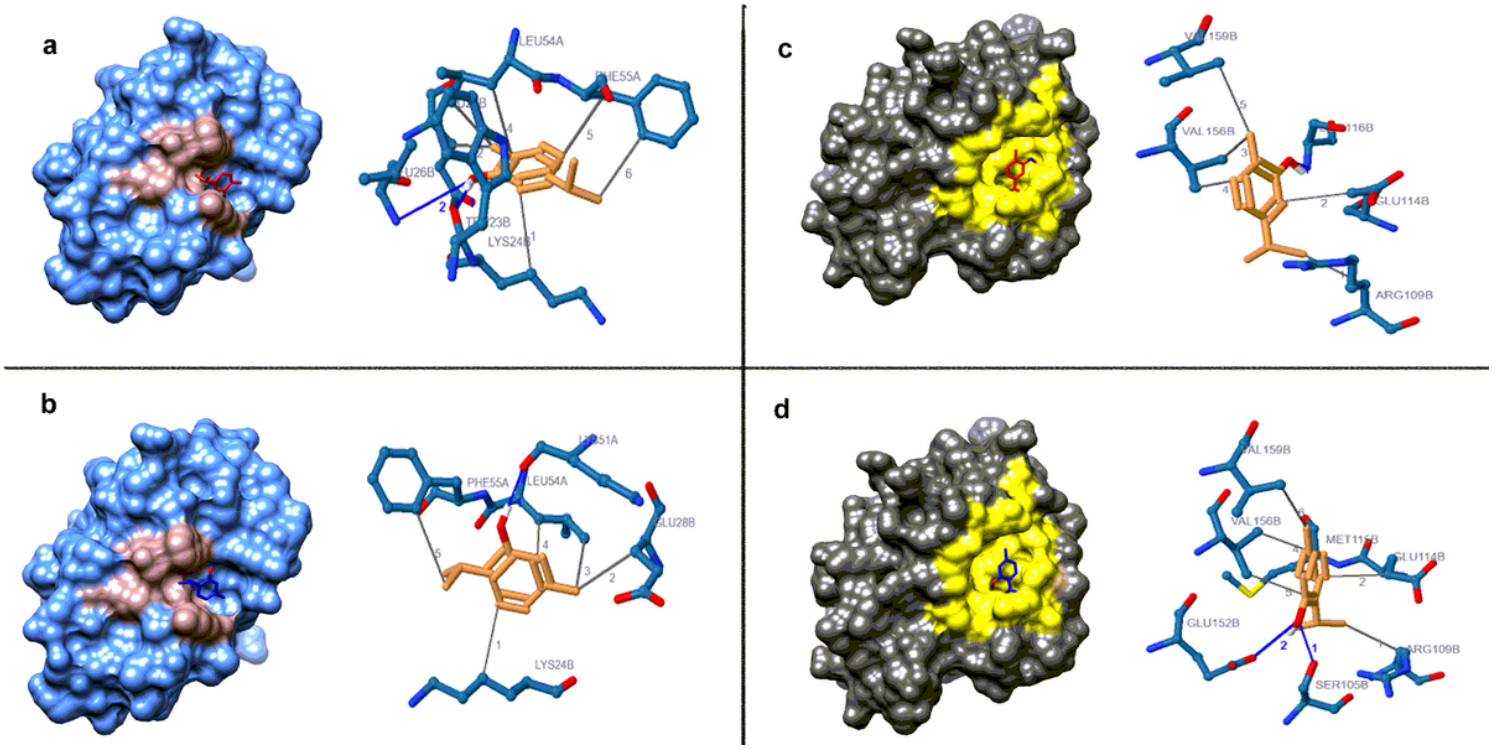

Figure 7

The 3D interaction and 2D interaction of P53 / Carvacrol (a), P53 / Thymol (b), BCL2 / Carvacrol (c) and BCL2 / Thymol (d). Pockets/ Cavities show the interaction of ligands with residues.