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IN VITRO SURVEY ON THE SYNERGISTIC EFFECT OF CICHORIUM INTYBUS L. AND DOXORUBICIN ON APOPTOTIC INDUCTION IN MYELOID (NALM-6) AND LYMPHOID (KG-1) CELL LINES

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Abstract – *Objective:* *Acute leukemia is a cancer of the blood and bone marrow. Due to chemotherapy drug side effect, the use of natural compound is important. The* Cichorium intybus *L is a natural compound which showed antitumor and antioxidant effect. The present study aimed to investigate the effect of the* C. intybus *extract, alone and in combination with Doxorubicin (DOX) on apoptosis induction in myeloid (NALM-6) and lymphoid (KG-1) cell lines.*

Materials and Methods: *In this* in vitro *experimental study, the NALM-6 and KG-1 cell lines were treated with different doses of* C. intybus *and DOX, and their combination for 24, 48, 72, 96 hours, and cell viability were investigated with the MTT assay. The percentage of apoptotic cells was determined using caspase-3, and 9 genes expression.*

Results: C. intybus *could induce cytotoxic effects in KG-1, Nalm-6 cell with IC50 values of 400±1.7 and 275±5.6 µg/ml. It has a significant apoptotic effect on Nalm-6 and KG-1 cell lines of ALL and AML in dose- and time-dependent manner (p<0.05). Gene expression of Bax and Caspase 3,and 9 increased and Bcl2 decreased (p<0.05).*

Conclusions: Cichorium *upgraded cytotoxic effect of DOX on Nalm-6 and KG-1 cell lines, and could be suggested as a chemotherapy supplement in acute leukemia.*

KEYWORDS: *Cichorium intybus, Acute leukemia, Apoptosis, CASPAS genes.*

INTRODUCTION

Acute leukemia (AL) is a heterogeneous malignancy of blood and bone marrow with a high prevalence (about 80%) in the age of 2-5 years[1]. AL refers to many immature white blood cells based on the type of stem cell involved, acute leukemia, including two main groups: AML (acute myeloid leukemia) and ALL (acute lymphoid leukemia). ALL is one of the most common malignancies among childhood, accounting for approximately 30% of pediatric malignancies. According to WHO classification, ALL is sub-divided into two groups: B-cell lymphoblastic leukemia/lymphoma and T-cell lymphoblastic leukemia/lymphoma[2,3]. The standard therapies for ALL include radiation therapy and chemotherapy, among which Doxorubicin (DOX) is a well-known chemotherapeutic drug[4]. DOX is a widespread anthracycline antibiotic to

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treat many malignancies such as breast, lung, stomach, bladder cancers, and Hodgkin's lymphoma[5]. DOX impairs DNA repair by inhibiting topoisomerase II and promoting cell death. Despite beneficial therapeutic outcomes, clinical administration of DOX is restricted because of remarkable toxicity[6,7]. Some studies have shown that natural compounds or herbal extracts such as Cichorium intybus L (C. intybus) can induce apoptosis in human breast cancer cells and could be helpful in cancer therapy in the future[8,9]. One of the most prominent plant families is Asteraceae which consists genus Cichorium with six members[10]. C. intybus is a wild, perennial herbaceous, deep-rooting, bright blue flower plant found in various world areas, including Africa, Asia, Australia, Europe, north and south America[11]. C. intybus contains linoleic acid and β-carotene with antioxidant properties, and Magnolialide, a 1β-hydroxyeudesmanolide with antitumor activities[12,13]. Other studies have confirmed the antitumor properties of C. intybus on several tumor cell lines[14]. Conforti et al[14] indicated that C. intybus extract induced an antiproliferative effect against amelanotic melanoma C32 cell lines. Migliorini et al[15] investigated the C. intybus effect on HepG2, HCT8, and Caco-2 cell lines and found decreased levels of ROS generation. They showed antioxidant, cytotoxic, and antiproliferative proprieties and attributed the antioxidant activity to high values of anthocyanin found in C. intybus exztract. Furthermore, in another study, Ebrahiminia et al[16] demonstrated that C. intybus leads to the differentiation of embryonic carcinoma stem cells into insulin-producing cells. They indicated this cell formed clusters similar to pancreatic islets with the molecular, cellular, and functional characteristics of mature β cells. Therefore, considering the importance of leukemia treatment and the need to find new options in this area, the present study aimed to investigate the effect of C. intybus and its combination treatment with Dox on Nalm-6 (ALL) KG-1 (AML) cell lines through CASPS gene expression.

MATERIALS AND METHODS

NALM-6 (ALL) and KG-1 acute leukemia (AML) cell lines were purchased from the National Cell Bank of Iran (NCBI). Fetal bovine serum (FBS) was purchased from Gibco Company (Denmark), and other chemicals such as MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], DMSO (dimethyl sulfoxide) were purchased from Sigma Company (Roedermark ,Germany).

Extract preparation

The C. intybus was purchased and authenticated by an academic expert. The aerial parts were dried and powdered. 400 g of dried powder were added to 800 ml of 70% ethanol, and the mixture was left to macerate at room temperature (percolation method) for 48 hours and then filtered through filter paper. The obtained extract was concentrated in the vacuum and was dried on the flat surface to evaporate alcohol solvent [17].

Cell culture

Two cell lines NALM-6 and KG-1, were cultured in RPMI-1640 and DMEM-F12 with 10% FBS, and penicillin 100 unit/ml, streptomycin 100 µg/ml were kept in standard condition (37°C humidified atmosphere with 5% CO_2). 10^4 cells were used in each well of 96-wells culture dish and were treated with extract (0, 50, 100, 200, 400 and 800 µg/ml) and Doxorubicin (0, 125, 250, 500 and 1000 nM/ml) for 24, 48, 72 and 96 hours. Six wells were used for each concentration in each espouser time.

MTT assay

Cell viability was determined with MTT assay. The cells were seeded in a 96 well plate (10^4 cells/well). The cell lines were treated with extract (0, 50, 100, 200, 400 and 800 µg/ml) and Doxorubicin (0, 125, 250, 500 and 1000 nM/ml). After treatments, the media were removed, and MTT solution (0.5 mg/mL) was added to each well and incubated for 4 hours. The formazan crystals produced by living cells were dissolved using DMSO (100 µL). Following the incubation for 15 minutes, the optical densities (OD) were measured using an ELISA reader at 570 nm and 630 nm. The percentage of cell viability was calculated according to the following formula:

$$\text{Cell viability (\%)} = [OD_{570,630}\,(\text{sample})/OD_{570,630}\,(\text{control})] \times 100$$

RT-PCR

RT-PCR assay was used to identify CASPASE-3 and 9 gene expressions. The cells were treated with the extract (0, 50, 100, 200, 400 and 800 µg/ml) and Doxorubicin (0, 125, 250, 500 and1000 nM/ml) that had the best IC50 on the MTT meth-

od for 48 h. The cells were collected, and their total RNA was extracted using TRIzol (Life Biolab, Germany). Then quantity and quality of extracted RNA were measured with NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All Total RNA extracted was saved at −80°C. 1µg total RNA was converted to cDNA using 2 step cDNA synthesis kit (BioFACT, South Korea) according to the manufacturer's instructions. The cDNA was stored at -20°C.

Real-time PCR assay

The effects of different doses of C. intybus on BAX, BCL-2, CASPASE 3 and 9 mRNA expression were measured by Real-time PCR. We use Master Mix SYBR Green I, High Rox (2x Real-Time Master mix, Bio FACT, South Korea). In this method which was performed by Applied Biosystems ™ Real-Time PCR instruments (Foster City, CA, USA). 10 µL of SYBR Green Master Mix, 2 µL of cDNA, and 200 nM of each primer were used in a 20 µL reaction mixture. Cycle conditions were as follows: denaturation at 95°C for 15 minutes followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Experiments were performed in triplicate to ensure reproducibility. The data were analyzed by the 2^-ΔΔCt method. In this method ΔΔCt: Sample ΔCt - Control ΔCt, Fold Change: 2^-ΔΔCt. The GAPDH gene was considered as the internal control. The primers were purchased from Cinagene Company (Tehran, Iran), and their sequences are reported in Table 1.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical evaluation was performed using two-way ANOVA analysis (GraphPad Prism 7 Software Inc, La Jolla, CA, USA).

TABLE 1. The primer sequences of genes

Gene Primer sequences
BAX F: 5′-cctgtgcaccaaggtgccggaact-3′
R: 5′-ccaccctggtcttggatccagccc-3′
BCL2 F: 5′-ttgtggccttctttgagttcggtg-3′
R: 5′-ggtgccggttcaggtactcagtca-3′
CASPASE-3 F: 5′-tggactgtggcattgagac-3′
R: 5′-caaagcgactggatgaacc-3′
CASPASE-9 F: 5′-catccttgtgtcctactccacc-3′
R: 5′-cagctttttccggaggaagt-3′

The primer Forward and Revers sequences of BAX, BCL2, CASPASE 3, and CASPASE-9 genes.

RESULTS

Cell Viability

The IC50 values of C. intybus were 400 ±1.7 µg/ml and 275 ±5.6 µg/ml for KG-1, Nalm-6 cell lines respectively, and it was 458 ±2.1 nM/ml and 239 ±3.04 nM/ml for DOX after 48 hr, respectively. Cell viability significantly reduced in KG-1 (p =0.05) and Nalm-6 (p =0.01) cell lines after 24, 48, 72, and 96 hr treatment with C. intybus (Figure 1) and Dox in that order (Figure 2) in a dose-dependent manner. The synergic effect of Dox and CIE on cell viability of KG-1 and Nalm-6 cells showed a significant (p<0.05) dose-dependent reduction (Figure 3).

Gene expression

The expression of the pro-apoptotic Bax gene increased in KG-1 cells after treatment with *C. intybus* and DOX alone. More effect is reported in combination treatment. Expression of antiapoptotic gene BCL2 reduced in KG-1 cells treated with C. intybus and Dox independently, and this reduction was more during the synergic treatment. In Nalm-6 cells, BAX gene expression increased after treatment with one of them. A higher effect was reported with combination treat, BCL2 gene expression levels decreased, and a synergistic effect was observed in the synergic group compared to the control group. To determine the apoptotic pathway induced by C. intybus and DOX alone, the expression of CASPASE-3 and 9 was measured. Results with the effect of C. intybus and DOX on cells showed the up-regulation of CASPASE-3 and 9 expressions in KG-1 and Nalm-6 cells treated with C. intybus and/or DOX compared to the control group, and more effect was indicated in the synergic group treatment (Figure 4).

DISCUSSION

The present study indicated that C. intybus has the apoptotic effect on Nalm-6 and KG-1 cell lines of ALL and AML in a dose- and time-dependent manner. Furthermore, C. intybus augmented the anti-proliferating effect of Dox on leukemia cell lines. A combination of C. intybus and Dox produced a significant decrease in cell viability of both cell lines. C. intybus, Dox, and their combination exerted a more potent effect on the Nalm-6 cell line compared to KG-1 cell line. Real-time results indicated the apoptotic and anticancer effect of C. intybus via increasing Bax, CASPASE 3 and 9, and decreasing Bcl2 gene expression.

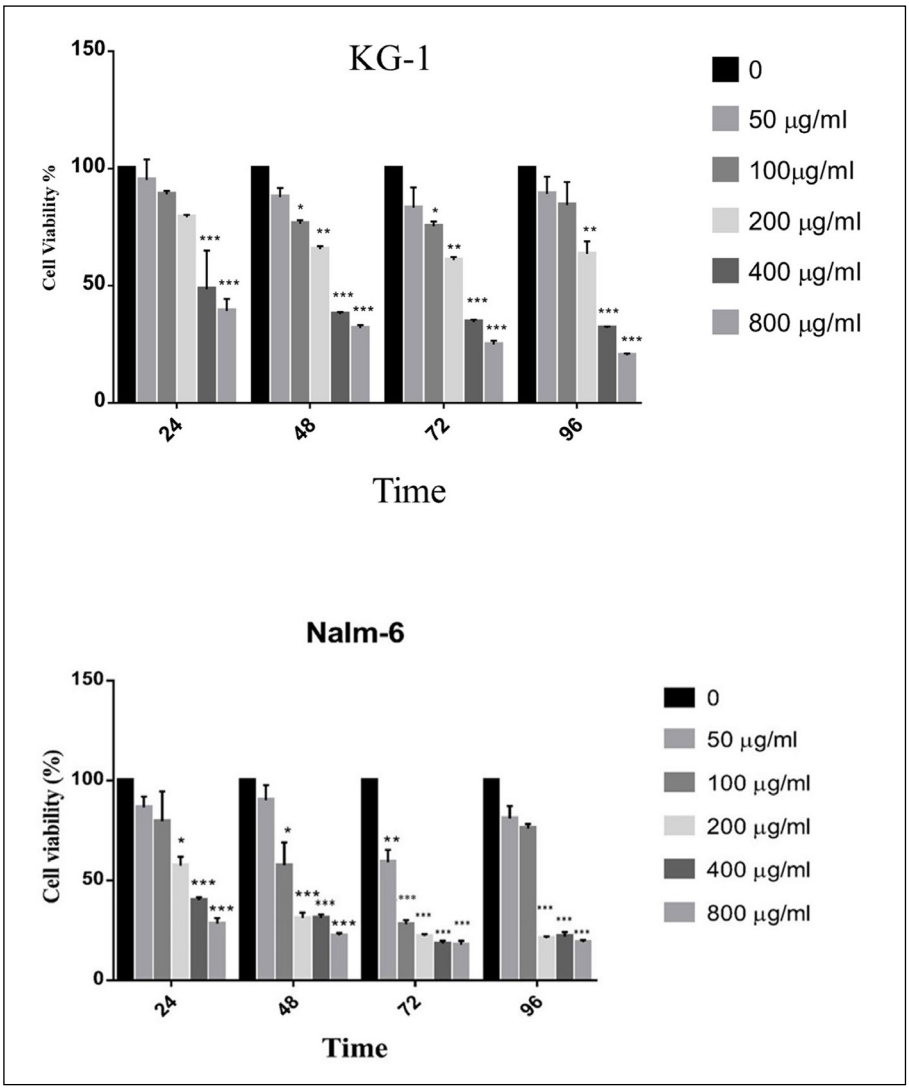

Fig. 1. The effect of C. intybus on cell viability of KG-1 and Nalm-6 cells. Cells were treated with different concentrations of C. intybus for 24, 48, 72, and 96 h. Cell viability was determined using the MTT assay as described in the methods section. Data are expressed in terms of percentage of control cells as the means ± SD (*: p <0.05, **: p <0.01: ***: p <0.001 compared with control, GraphPad Prism 7 Software Inc. (La Jolla, CA, USA).

There are several treatments for leukemia. The most common is chemotherapy using DOX (an anthracycline agent isolated from Streptomyces peucetius in the 1960s) [18], which is also used to treat ALL and AML. Like more chemotherapy drugs, DOX has side effects including anemia, vomiting, low platelet count (thrombocytopenia), inflammation of the mouth, congestive heart failure (CHF) and bone marrow suppression, and cytotoxic effect [19]. Today, much evidence proposing herbal-derived dietary compounds and supplements show anticancer and apoptotic potential alone and combined with chemotherapy drugs.

One of the suggested herbs for cancer treatment in the traditional medicine of different countries (Romans and Ancient Egyptians) is Cichorium[12,20]. C. intybus is used in various illnesses like liver diseases, malaria, diarrhea, diabetes, pulmonary disease, and prostate cancer[21]. Our previous study showed different concentrations of C. intybus significantly improved oxymetholone-induced liver changes, including serum biochemical and histological changes[17]. In the present study, we used a hydroalcoholic extract of the aerial part of C. intybus on Nalm-6 and KG-1 cell lines of ALL and AML. Some studies indicate that C. intybus contains various phytochemicals such as anthocyanin, sterol, phytosterol, Caffeic acid, guaianolides, 6methoxy flavone, eudesmanolides, germacranolides, polyacetylene, delphinidin, 3, 4-dihydroxyphenethyl, 1, 3-dicaffeoylquinic acid, and cyanidin-3-O-galactoside[22-24] which explain Chicory anticancer and anti-inflammatory effect.

Rahimipour el al[25] showed a methanolic extract of C. intybus reduced the cell viability of breast cancer cells (SKBR3) in a time-dependent manner; also, we observed the same effect on ALL and AML cell lines. In a study on lymphocytic leukemia Jurkat cells, it was demonstrated that the aerial part of

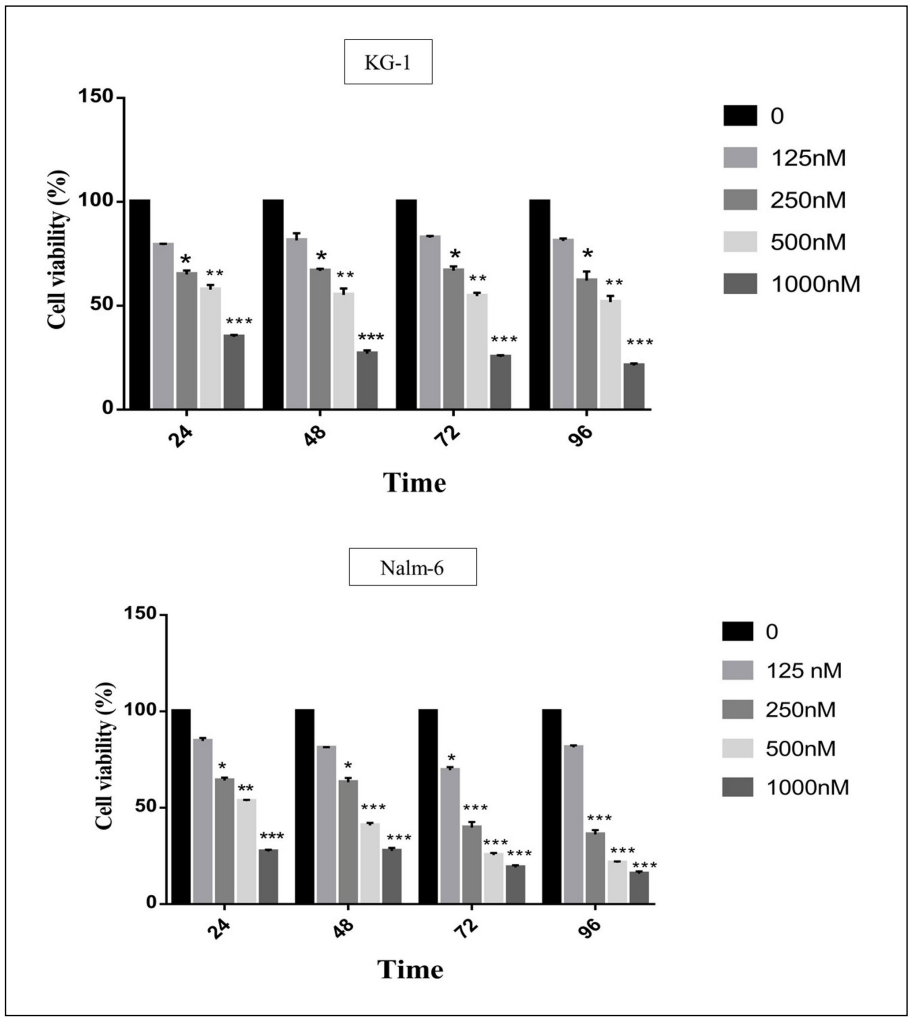

Fig. 2. The effect of Dox on cell viability of KG-1 and Nalm-6 cells. Cells were treated with different concentrations of Dox for 24, 48, 72, and 96 h. Cell viability was determined using the MTT assay as described in the methods section. The data are expressed in terms of percent of control cells as the means ±SD (*: p <0.05, **: p <0.01; ***: p <0.001 compared with control, GraphPad Prism 7 Software Inc.).

C. intybus showed significant antiproliferative effect (70% at 100 µg/ml) as well as cytotoxic activity (50.3% at 100 µg/ml)[26]. We confirmed the same inhibitory effect in the present study. In similar studies, Caffeic acid compound derived from the leaf of C. intybus was highly effective at human promyelocytic leukemia cells (HL-60; 90% inhibition at 10 µM)[27,28] so we can refer some Chicory effects to Caffeic content of C. intybus. *In vivo* studies by Hafez et al[28] indicated that whole-plant extracts of C. intybus lowered the expression of natural interferon α (INF-α) and B-cell lymphoma 2 (Bcl-2), supporting the Cichorium antitumor property. Another study found tumor growth inhibition in the mouse model of colorectal cancer after being fed with whole-plant water extract[29]. An investigation attributed the anticancer and antiproliferative effect of C. intybus to phytochemicals such as 1, 3-dicaffeoylquinic acid, 3, 4-dicaffeoylquinic acid, cyanidin-3-O-galactoside[30,31]. A study on 1,3-Dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid demonstrated depressed cancer cell proliferation (colon cancer DLD-1 cells)[26].

Rosa et al[32] reviewed phytochemicals (cyanidin-3-O-galactoside and Caffeic acid) effects on the alteration of cellular signaling pathways, DNA damages repair, cell proliferation, apoptosis, invasion, and gene expression regulation. Same as some parts of our study, Cho et al. investigated cyanidin-3-O-galactoside effect on MDA-MB-453 cells and *in vivo* model, they indicated that Bax and CASPASE 3 expression increased in contrast to decreased Bcl2 expression. These results showed that cyanidin-3-glucoside induced apoptosis in breast cancer (MDA-MB-453) cells, suppressed proliferation, and growth *in vitro* and *in vivo* model also decreased cell viability via alteration of apoptotic protein contents and indicating the inhibition of tumor progression[33].

In a study on human leukemic HL-60 cells, Chen et al[27] showed caffeic acid is a potent apoptosis-inducing agent by increasing CASPASE 3 expression, Bax up-regulation, and Bcl-2 down-regulation. Also, they suggested that this compound induced DNA fragmentation, morphological changes, and induced apoptosis in these cells. In

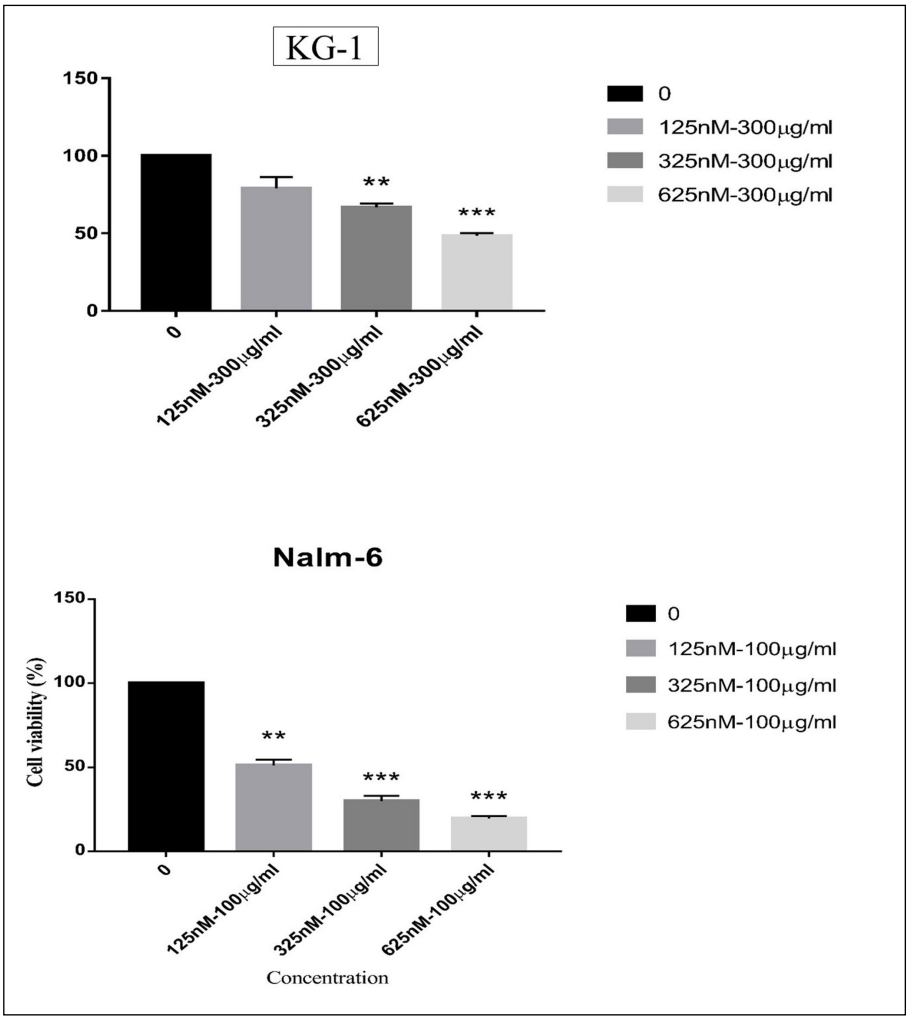

Fig. 3. The synergic effect of DOX (125, 325, and 625 nM) and C. intybus (100 µg/ml) on the cell viability of KG-1 and Nalm-6 cells. Cells were treated with indicated concentrations of extract for 24, 48, 72, and 96 h. Cells viability was determined using the MTT assay as described in the methods section. The data are expressed in terms of percent of control cells as the means ±SD (**: p <0.01; ***: p <0.001 compared with control, GraphPad Prism 7 Software Inc.).

another study, Kinjo et al[34] indicated much more potent inhibitors of HeLa cell growth by 6 methoxy flavone. Therefore, the 6-methoxy group is likely important for enhancing the antiproliferative activity of flavones against HeLa cells.

Two major phytosterols found in C. intybus leaves are Campesterol and Phytosterol/sterol. A study demonstrated antitumor and antiproliferative proprieties of Campesterol against human liver cancer (HepG2) and human breast cancer cells (MCF-7) [35]. Ryan et al[36] investigated the effect of phytosterol and sterol compounds on three cell lines (human monocytic cell line (U937), colonic adenocarcinoma cell line (CaCo-2), and hepatoma liver cell line (HepG2), showing apoptotic effects on U937 cell line via Bax, and CASPASE-3 up-regulation.

A study investigated the toxicological effect of C. intybus root extract in 28 days in an animal model (female Sprague-Dawley rats) and found no adverse effects at 1000 mg/kg/day dose[37]. Some clinical trial studies investigated the C. intybus effect on different diseases. In the first study, Schumacher et al[38] in patients with osteoarthritis showed administration of osteoarthritis. In the second study, Olsen et al[39] indicated C. intybus combined with coffee could reduce whole blood and plasma viscosity and decrease cardiovascular disorder. In another clinical trial study in cirrhotic patients, Huseini et al[40] showed anti-inflammatory, antioxidative, hepatoprotective effects and immunomodulating properties of C. intybus that improve cirrhotic patients.

CONCLUSIONS

Our results showed that C. intybus enhanced DOX cytotoxic effect on Nalm-6 and KG-1 cell lines and could be suggested as a chemotherapy supplement in acute leukemia. More clinical trial studies are needed to confirm the application of our findings in leukemia patients.

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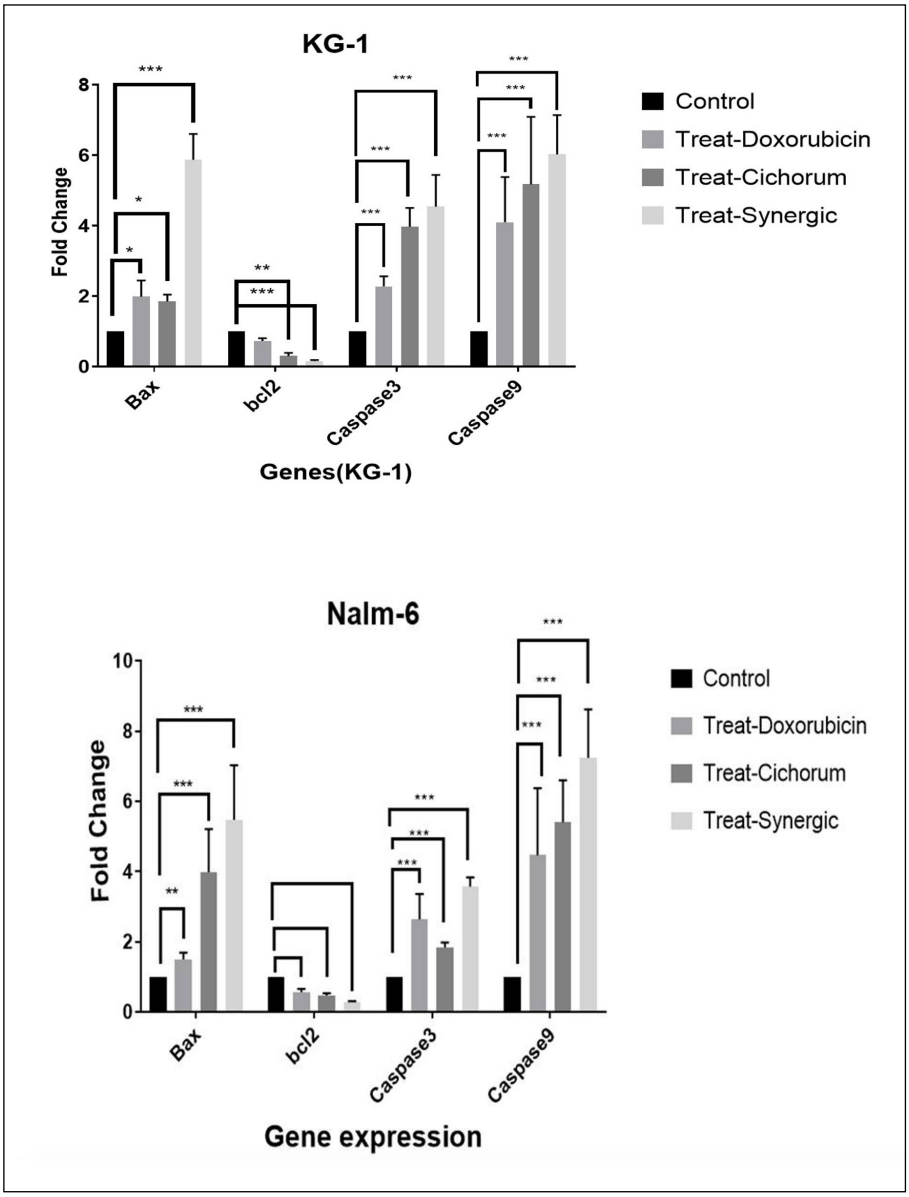

Fig. 4. The expression level of BAX (pro-apoptotic), BCL-2 (antiapoptotic), and CASPASE-3 (required enzyme for the execution of apoptosis), 3 and 9 (initiator caspase) genes in KG-1 and Nalm-6 cells after treatment with C. intybus, Dox, and synergic for 48 h was evaluated by real-time PCR. The data are expressed in terms of percent of control cells as the means ±SD (*: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$) compared with control, two-way ANOVA with GraphPad Prism 7 Software Inc.).

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Conflict of Interest:

The authors declare that they have no competing interests. This manuscript has not been published elsewhere, and it has not been submitted simultaneously for publication elsewhere.

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