

Research Article

# Combination Effect of Broccoli Extract and Doxorubicin on Cell Proliferation and Cell Cycle Arrest in LNCaP Prostate Cancer Cell Line

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## ABSTRACT

**Background:** Several studies have documented that phytochemicals in Broccoli extract (cruciferous) contain cancer-preventive activity. It was shown that this extract can kill cancer cells without any effect on normal cells. The aim of the present study is to investigate the effect of broccoli extract, doxorubicin (Dox) and their combination on cell viability and cell cycle arrest in prostate cancer model. **Methods:** Crude broccoli extracts was educed with 95% ethanol. LNCaP cells were treated by different concentration of the broccoli extract and Doxorubicin for 24 and 48 hours. The cells' proliferation rate was assessed using MTT assay. The apoptotic cells were observed morphologically using Acridine orange/ Ethidium bromide double staining and cell cycle analysis was done with flowcytometry. **Results:** The results showed that both DOX and the crude extract of broccoli represented cytotoxic effects on prostate cancer cells, LNCaP, after 24h. Combination effects of these agents also decreased cells' proliferation rate compared to the control group and the drugs alone. In the combination mode the amount of Dox as a chemical drug containing side effects, reduced significantly. Flowcytometric analysis of the cell cycle showed that the broccoli extracts, Dox and their combination caused increasing the number of cells in sub-G1 phase and G2/M arrest. In addition, Acridine orange/ Ethidium bromide staining revealed morphological changes of apoptosis in the treated cells. Therefore, it seems that crude extract of broccoli included apoptotic effects and could potentiate Dox effects. **Conclusion:** The present finding showed the broccoli extract potential to induce apoptosis and cell cycle arrest in cancer cells. This may be used in cancer therapy to reduce chemical drug doses and sides effects.

**Keywords:** Apoptosis, Broccoli, Cell Cycle, Doxorubicin (Dox), LNCaP Prostate Cancer Cell line

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## INTRODUCTION

The prostate cancer (PC) is the second cause of death after lung cancer and causes over one third of deaths in the world<sup>1</sup>. PC is a multi-stage pathogenesis because genetic and epigenetic changes resulting in cell imbalances in proliferation, apoptosis, and differentiation<sup>2</sup>. Although the early diagnosis of the prostate cancer became somehow possible upon discovery of the prostate-specific antigen, the available treatments are still limited<sup>3-5</sup>. The treatments such as the surgical removal of the testes and anti-androgenic therapy often inhibit the early stages of tumor development<sup>6</sup>. However, the tumor relapses in many cases showed the necessitate of secondary hormonal therapy and cytotoxic chemotherapy<sup>7-9</sup>. The chemotherapies are generally experimental and used as a combination of chemotherapeutic drugs based on their efficiency. Despite chemotherapeutic widespread use, there is not enough experimental information in this regard<sup>10-13</sup>. A known chemical drug is doxorubicin, an anthracycline antibiotic works by intercalating DNA in G2 phase of the cell cycle<sup>14,15</sup>. Doxorubicin has extensive effects on solid tumors and hematological malignancies but with cardiac toxicity and myeloid suppression effects<sup>16-19</sup>.

Epidemiological studies showed that consumption of plant-based diets rich in phytochemicals, such as grains, vegetables, and fruits can reduce the risk of certain cancers, such as prostate<sup>20-22</sup>. The cruciferous vegetables like broccoli have been studied in terms of their anti-carcinogenic properties<sup>23</sup>. The anti-carcinogenic and chemo-protective properties of this family of vegetables mainly are largely attributable to the dietary intake of glucosinolates which are transformed into isothiocyanate metabolites in the body by the enzymatic action of plant specific myrosinase and/or gut microflora<sup>6</sup>. A large part of anti-carcinogenic property of broccoli associates with an enzyme that upregulates phase II detoxification enzymes. It might results in clearance of carcinogenic substances and reactive oxygen species (ROS)<sup>24</sup>. In the present study we supposed that broccoli could enhance the doxorubicin anti-proliferative effects. Therefore, we examined anti-proliferative and apoptotic effects of the broccoli extract and Dox combination in a cellular model of prostate cancer.

## MATERIAL AND METHODS

### Cell Culture

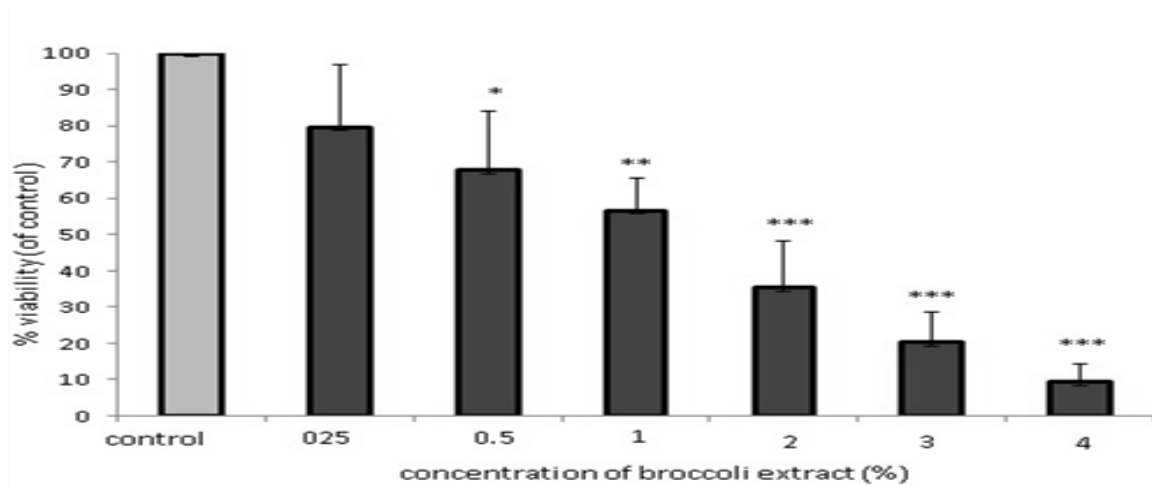


Figure 1: The effect of the aqueous extract of broccoli on LNCap cells for viability. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ ).

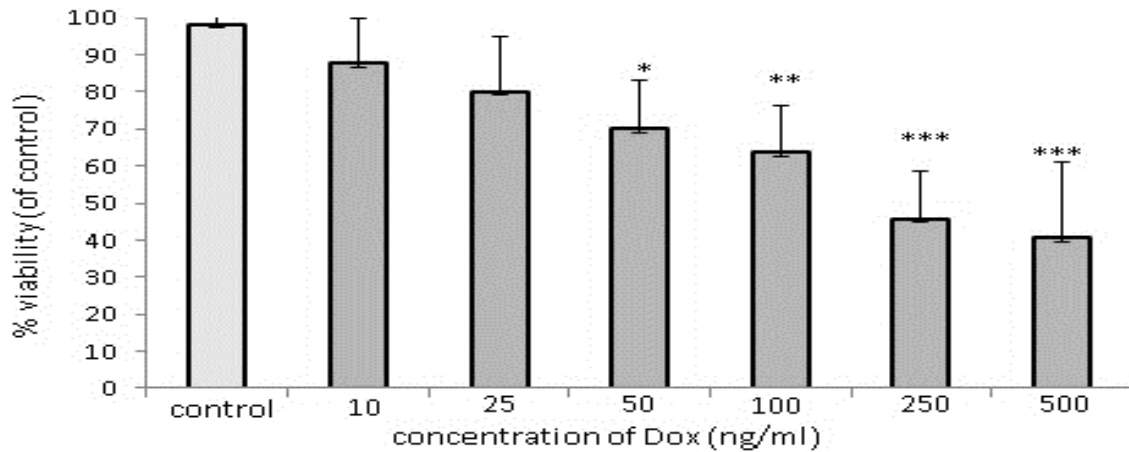


Figure 2: The effect of DOX on LNCap cells viability after 24h (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ ).

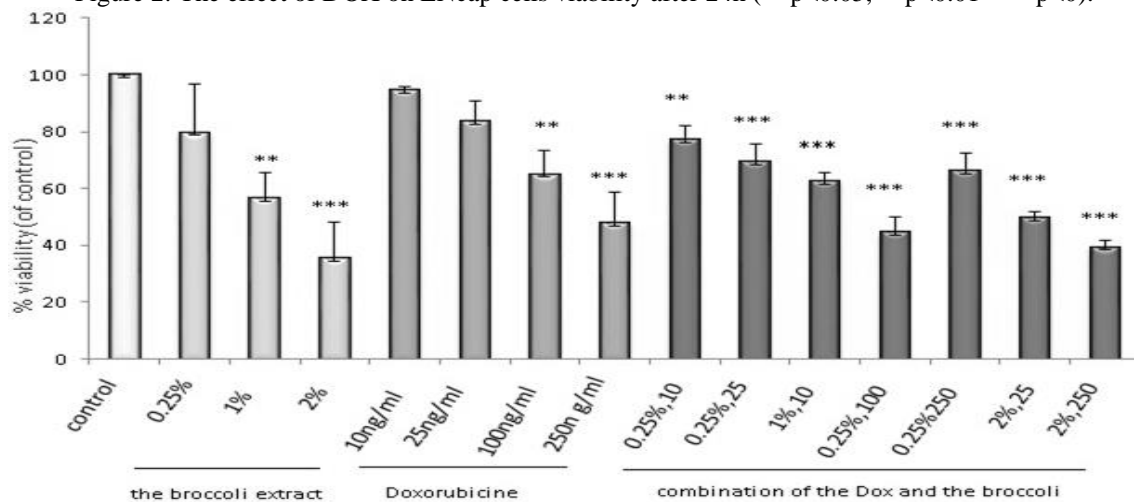


Figure 3: The effect of the combination of the Broccoli extract and Dox on the LNCap cells viability after 24h (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ ).

The human LNCaP cells were obtained from National Cell Bank of Iran (NCBI). The cells were cultured in RPMI1640 (Gibco) supplemented with 10% of heat inactivated fetal bovine serum (FBS) (Gibco), L-glutamine 1%, penicillin 100 units/ml and streptomycin 100 mg/ml, at 37 °C and 5% CO<sub>2</sub> in a humidified atmosphere.

#### Broccoli Extract Preparation

30 g of dry broccoli powder was poured in an Erlenmeyer flask, and 100 ml of sterile distilled water with 70-80°C was added to the flask. Then, the flask was placed in a 60°C bath water. After 24 hours, the solution was

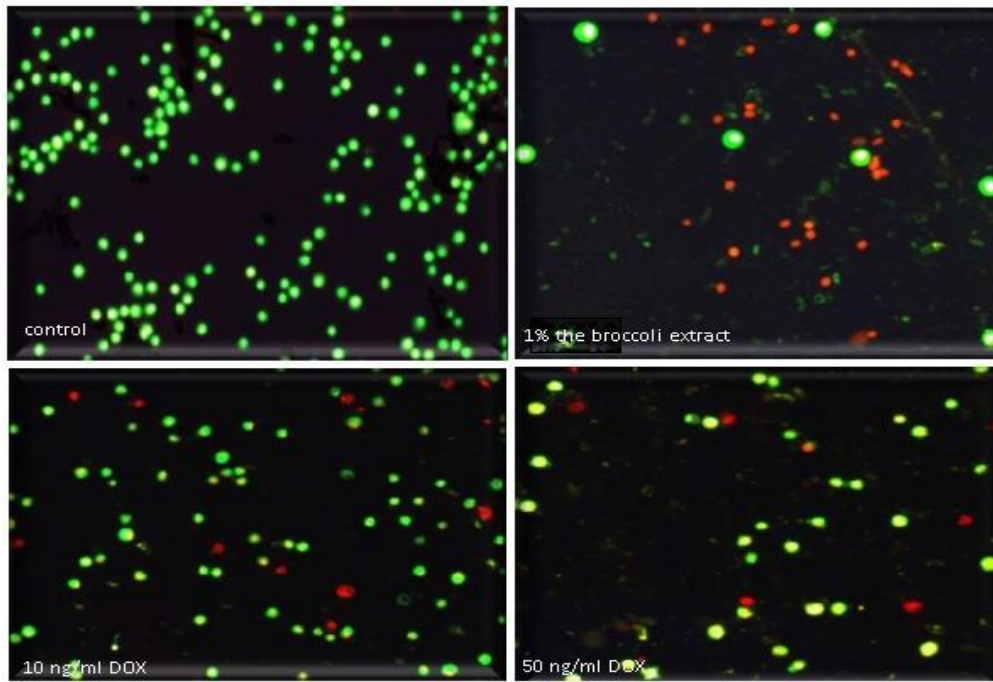


Figure 4: Morphological study of LNCap cells by AO/EB staining treated with Dox, the broccoli extract and their combination for 24h.

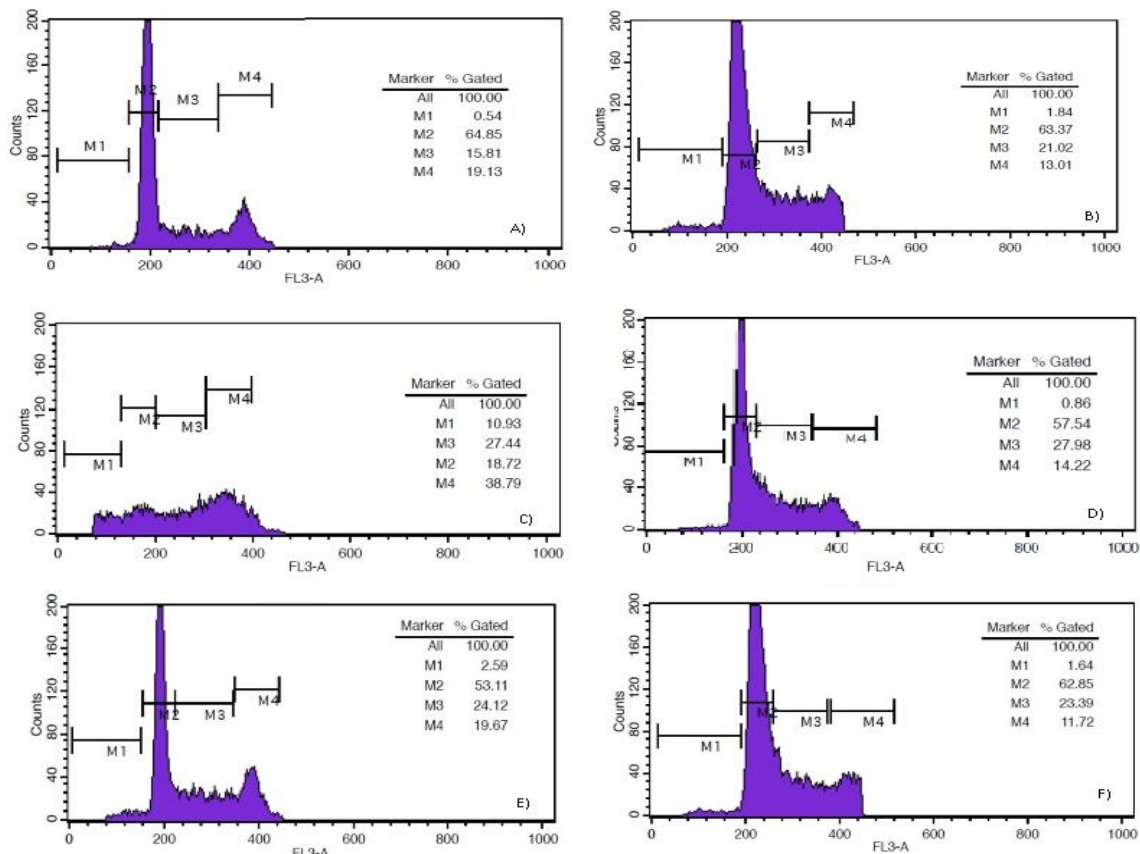


Figure 5: Histogram of Cell cycle analysis of LNCap cells treatment with broccoli extract, DOX and their combination by flowcytometry. A) Control group, B) 0.25% Broccoli extract, C) 2% Broccoli extract, D) 25ng/ml DOX, E) 250ng/ml DOX, F) noneffective combination of the drugs. (M1: sub-G1 area, M4: G2/M phase).

concentrated, and the obtained extract was filtered using filter paper and Buchner funnel.  
*Treatment of the Cells*

To determine the effect of different concentrations of the extract on LNCap cells,  $1 \times 10^4$  cells were cultivated in 96-well plates. After 24 hours, the cells' media was removed,

Table 1: The effect of the broccoli extract, DOX and their combination on cell cycle analysis in LNCaP cells for 24 hour (\*p<0.05, \*\*p<0.01, \*\*\* p<0.0001).

	SubG1	G1	S	G2
control	0.54	64.75	15.81	19.13
0.25% Broccoli	1.84	63.37	13.5	23.01
2% Broccoli	10.93***	18.72***	27.44***	35**
25ng/ml DOX	0.86	57.74	15.5	14.22
250ng/ml DOX	2.59*	53.11***	14	57.87***
0.25% B+25ng/ml D	1.64	62.85	25.39**	14.2
0.25% B+250ng/ml D	3.28*	54.99**	16	35
2% B+25ng/ml D	4.55***	56.92	24**	18.5
2% B+250ng/ml D	9.11***	31.03***	22*	38.5**

and fresh media containing different concentration of the broccoli extract (0.25, 0.50, 1, 2, 3, and 4%), Dox (10.25, 50, 100, 250 and 500 nM/ml) and their combination were added to the cells for periods of 24 hours.

#### MTT Cytotoxicity Assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma–Aldrich) is a yellow substrate that could enter viable cells and transform to a dark blue product called formazan. The LNCaP cells were maintained in the exponential growth phase and treated with different concentrations of the broccoli extracts, Dox or combination of both. After 24h MTT to a final concentration of 1mg/ml was added and the cells were incubated for 4 hours. The formazan product in viable cells was resuspended in Dimethyl sulfoxide (DMSO- Merck) to dissolve and the absorbance was measured at 630nm. The required concentration necessary to produce 50% cell growth inhibition (IC50) was determined by interpolation from dose-response curves.

#### Acridine Orange/Ethidium Bromide (AO/EB) Dual Staining

Morphological features of apoptotic and necrotic cells were determined by EB/AO dual staining and the incidence of normal, apoptotic and necrotic cells was obtained by counting. The number of apoptotic, necrotic and normal cells was determined by counting using a Whipple Reticles. Following the cells treated with drugs, the cells were detached and washed with PBS. The cell suspension then mixed with AO/EB solution (Merck) in a final concentration of 100µg/ml and the cells were analyzed under fluorescence microscope (Zeiss) and photographed.

#### Cell Cycle Analysis

Upon collection of the cells treated with the drugs, the LNCaP cells washed with PBS, then 50 µl of cold PBS was added to the cells and slowly stirred by vortex. Then, 1 ml of the cold 70% ethanol was added to the samples and fixed. After that, the cells were mixed with 1 ml of propidium iodide (PI) master mix solution containing 40 µl of PI (Merck), 10 µl of RNase I (Fermentas), and 950 µl of PBS and finally, the cells were incubated for 30 min at 37°C and analysis using flow cytometry. The data were analyzed using WINMDI software.

#### Statistical Analysis

The statistical analyses were performed using SPSS software and the non-parametric one-way ANOVA. The

differences were significant at  $P \leq 0.05$ , and the results were shown as mean values.

## RESULTS

### Cytotoxic Effect of Crude Broccoli Extracts on LNCaP cells

The broccoli crude extract significantly reduced viability rate of the cells in 24 hours (Fig. 1). Compared to the control group, viability percentage decrease of cells occurred at 0.50% ( $P < 0.05$ ); 1% ( $P < 0.01$ ); 2%, 3%, and 4% ( $P < 0.001$ ) concentrations of the broccoli extract. Therefore, it could be seen that the effect of the aqueous broccoli extract on LNCaP cells was in a dose dependent manner within 24 hours. The IC50 (The half maximal inhibitory concentration) was observed about 1.2% w/v of the broccoli extract after 24h of treatment.

### Cytotoxic Effect of Doxorubicin on LNCaP cells

After incubation with 10 ng/ml and 25 ng/ml DOX for 24 h, a significant decrease in cells viability was detected in the samples. Moreover, the 50 ng/ml concentration reduced the viability percentage of cells to 74% ( $P < 0.05$ ) in the 24-hour treatment. However, concentrations of 100 ng/ml, 250 ng/ml, and 500 ng/ml in 24-hour were 69%, 51%, and 47%, respectively significantly reduced the viability percentage of cells than that in the control group ( $P < 0.001$ ) (Fig. 2). The IC50 for Dox was about 100 ng/ml after 24h.

### Cytotoxic effects of combination of broccoli extract and doxorubicin on cells viability

Using effective and ineffective concentrations (IC50 and higher concentrations) of doxorubicin in the presence of various concentrations of the broccoli extract was examined on the LNCaP cells in a 24- hour duration (Fig. 3). Co-treatment with the broccoli extract and DOX significantly increased cytotoxicity in treated cells compared with controls ( $P < 0.001$ ). The combination of effective dose of DOX 250 ng/ml and none effective concentration of the broccoli extract 0.25% caused 26% and 45% killing after 24 h. Furthermore, combination of none effective dose of DOX (25 ng/ml) and effective concentration of the total broccoli extract (2%) caused 51% of cells killed after 24hours. Co-treatment of cells with effective doses of DOX and the broccoli extracts killing rate was 61% and 39% after periods of 24 and 48-hours. These results demonstrated that combination of DOX with the total broccoli extract was can improve the

effect of DOX none effective concentration in LNCaP cells.

#### *Acridine Orange/Ethidium Bromide Double Staining*

Morphological changes in nuclei such as nuclear segmentation is an early hallmark of apoptosis, which may result in cell death. Acridine orange/ Ethidium bromide (AO/EB) double staining test is fast and statistically significant fluorescence method to determine the morphological changes in nuclei, cell viability and distinguish the viable apoptotic and necrotic cells from early and late stage<sup>25</sup>. To determine morphological changes of the cells, AO/EB staining followed fluorescence microscopy analysis were done. Figure 4 shows data from AO/EB staining of LNCaP cells treated with DOX and the total broccoli extract under different treatment condition. The rate of viable cells reduced by increasing concentration of the drugs. In control groups, the normal cells were satin green. Whereas the early apoptotic cells as bright green nucleus and the late apoptotic cells with condensed and fragmented orange chromatin were observed after treatment with DOX, the broccoli extract and their combination. In the combination of the drugs, it seems the total broccoli extract contained the capacity to improve DOX effect in its noneffective concentration (Fig. 4).

#### *Cell Cycle Analysis*

The flowcytometric analysis of cell cycle was done in order to determine whether the DOX and the aqua broccoli extract induced inhibition of cell proliferation (Fig. 5). At the late stage of the apoptotic cascade, endonucleases break the linkers between the nucleosomes, and large numbers of small fragments of DNA, whose sizes are about 180 bp, accumulate in the cell. If cells are fixed in ethanol some of the lower molecular weight DNA leaches out, lowering the DNA content. These cells can be observed as a hypodiploid or 'sub-G1' peak in a DNA histogram<sup>26</sup>. It was showed that noneffective concentration of the broccoli extract (0.25%) did not change the percentage of cells in the sub-G1-phase population, while IC50 concentration of the broccoli extract caused the rate of cells in sub-G1-phase increased from 26% to 43% after 24 h ( $P < 0.001$ ). In doxorubicin treated cells the sub-G1-phase population increased from 18% to 39% ( $P < 0.001$ ). The percentage of cells in the sub-G1-phase population increased in the combination of the noneffective concentrations of the broccoli extract (0.25%) and doxorubicin (25 ng/ml) although such increasing was not significant. However, the concurrent treatment with the same concentration of the broccoli extract (0.25%) and the effective concentration of doxorubicin (250 ng/ml) significantly increased the sub-G1-phase population ( $P > 0.001$ ). Moreover, the combination of the effective concentration of the broccoli extract (2%) and ineffective concentration of doxorubicin (25 ng/ml) increased the sub G1-phase population ( $P > 0.01$ ) in a way that the 25 ng/ml concentration of doxorubicin could not significantly change the cell cycle population (18%). Such an increase was also evident for the effective concentration of doxorubicin (250 ng/ml) in combination with the 2% of the broccoli extract which increased from 39% to 47%

( $P < 0.001$ ). The quantitative data of the cell cycle analysis are shown in table 1.

#### **DISCUSSION**

Recent studies have indicated that diet has a major role in the development and pathogenesis of the most diseases like cancer and many conventional antitumor drugs are derived from natural products<sup>27</sup>. It has been shown that fruits and vegetables can promote cancer therapy<sup>28</sup>. The cruciferous family vegetables can help decreasing the risk of developing cancer and other degenerative diseases<sup>29</sup>. Broccoli is a valuable source of glucosinolates and isothiocyanates like sulforaphane. In recent years, broccoli has been an increasing interest for its therapeutic and preventive effects on prostate cancer<sup>30-33</sup>. The present study was designed to determine whether the combination of broccoli extract and doxorubicin induce apoptosis or inhibit cell growth process in a cellular model of prostate cancer. Doxorubicin is an antitumor drug that has been widely used for treatment of several cancers including prostate cancers<sup>34</sup>. The effect of the broccoli extract on human carcinoma cells showed that this extract strongly inhibited carcinoma cell growth *in vitro*<sup>35</sup>. It has been also revealed that doxorubicin combination with drugs such as zolidonic acid could increase the anticancer effects of doxorubicin in an *in vitro* model of prostate cancer<sup>36</sup>. The present data showed that both doxorubicin and the broccoli extract caused cytotoxicity effects on LNCaP cells in a concentration dependent manner which is due to reduction of cell proliferation efficiency after 24h. One of the interesting finding is that none-effective dose of doxorubicin demonstrated inhibiting effects on cell growth when applied in combination with the broccoli extracts. The co-treatment of the non-effective concentration of the broccoli extract with the non-effective concentration of DOX significantly reduced the viability percentage of LNCaP cells than the control group. Furthermore, the effective concentration of the broccoli extract in combination with the non-effective concentration of DOX also significantly reduced the viability percentage in LNCaP cells. Surprisingly, the main findings regarding combined effect of the broccoli extract and Dox on growth inhibition was about the cell cycle alteration. Non-effective dose of Dox combined with the IC50 concentration of the broccoli extract caused significant increase of cell population in sub-G1 phase. In addition, co-treatment of the cells with the both non-effective concentrations of the drugs resulted in G2/M arrest. These results are in line with previous studies showed that the broccoli extract contained anti-proliferative activity in another prostate cancer cell line PC3 which is associated with apoptosis induction<sup>37</sup>. Other studies have found that the broccoli derivatives such as suloraphane reduced growth of human PC-3 prostate xenografts in nude mice<sup>38</sup>. As most of the current studies tend to examine the combined treatment for cancer, it seems our results are to be consistent with this goal and it can thus be suggested that the combination of broccoli extract and doxorubicin decreases doses of this drug used in the treatment and the resulting side effects. It has been demonstrated that the

broccoli extract enriched with selenium, could fort chemosensitivity and apoptosis in LNCaP prostate cancer cells<sup>4</sup>. Based on the results, it can be concluded that the combination of doxorubicin and broccoli extract can potentiate the effectiveness of doxorubicin and reduce the dose used in the treatment. The previous studies revealed that components of broccoli such as sulforaphane increased the efficiency of doxorubicin in cases of drug resistance and improve the induction of apoptosis in cancer cells through the activation of p53 in the intrinsic pathway<sup>39</sup>. The analysis of the cell cycle changes confirmed that combination of the DOX and the broccoli extract induced apoptosis in LNCaP cells as the combination resulted an increasing in the number of the cells which are in sub-G1 phase of the cell cycle. Indeed, co-treatment of the cells with non-effective doses of the both drugs caused a G2/M arrest. These data indicated that apoptotic events should be under presence of DOX and the broccoli extracts and it can be concluded that the apoptotic effect of doxorubicin increases when combined with broccoli. Therefore, combining the broccoli extract with doxorubicin maintains the effectiveness of doxorubicin effects besides increasing the apoptotic induction through raising the percentage of subG1 phase. According to the results of this study, the important point was that the combination of the non-effective dose of doxorubicin and the broccoli extract showed a decrease in cell viability percentage, an increase in sub-G1 area of cell cycle, and also a high arrest in G2/M phase significantly. Based on the results, it can be concluded that the concurrent use of doxorubicin and broccoli extract or its ingredient such as sulforaphane can reduce the dose of doxorubicin used in the treatment, and consequently, reduce side effects of this chemotherapeutic drug.

#### CONFLICT OF INTEREST.

There is no conflict of interest between authors

#### ACKNOWLEDGMENT

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