Original Article

Effects of Combined Soy Isoflavone Extract and Docetaxel Treatment on Murine 4T1 Breast Tumor Model

Ehsan Hejazi [1], Javad Nasrollahzadeh [1], Ramina Fatemi [2], Leila Barzegar-Yar Mohamadi [3], Kioomars Saliminejad [4], Zohre Amiri [5], Masoud Kimiagar [1], Mohammad Houshyari [6], Maryam Tavakoli [2*], and Farah Idali [2*]

1. Department of Clinical Nutrition and Dietetics, School of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Reproductive Immunology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
3. Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
4. Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
5. Department of Basic Sciences and Cellular and Molecular Nutrition, School of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
6. Department of Radiation Oncology, Shohada Tajrish Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*** Corresponding authors:**
Farah Idali, Ph.D., and Maryam Tavakoli, M.D., Ph.D., Reproductive Immunology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
Tel: +98 21 22432020
Fax: +98 21 22432021
E-mail:
F.Idealı@avicenna.ac.ir;
Tavakoli@avicenna.ac.ir
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Abstract

Background: Emergence of drug resistance has brought major problems in chemotherapy. Using nutrients in combination with chemotherapy could be beneficial for improvement of sensitivity of tumors to drug resistance. Soybean-derived isoflavones have been suggested as chemopreventive agents for certain types of cancer, particularly breast cancer. In this study, the synergistic effects of soy isoflavone extract in combination with docetaxel in murine 4T1 breast tumor model were investigated.

Methods: In this study, mice were divided into 4 groups (15 mice per group) of control, the dietary Soy Isoflavone Extract (SIE, 100 *mg/kg* diet), the Docetaxel (DOCE, 10 *mg/kg*) injection and the combination of dietary soy isoflavone extract and intravenous docetaxel injection (DOCE+SIE). After 3 injections of docetaxel (once a week), 7 mice were sacrificed to analyze MKI67 gene and protein expressions and the rest were monitored for diet consumption, tumor growth and survival rates.

Results: In DOCE+SIE group, diet consumption was significantly higher than DOCE group. While lifespan showed a trend towards improvement in DOCE+SIE group, no significant difference was observed among the 4 studied groups. Tumor volume was not significantly affected in treated groups. A lower but not significant MKI67 protein expression was detected in western blot in DOCE+SIE group. The mRNA expression was not significantly different among groups.

Conclusion: The results suggest that the combination of soy isoflavone as an adjunct to docetaxel chemotherapy can be effective in improving diet consumption in breast cancer.

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Keywords: Breast cancer, Docetaxel, Soy isoflavone extract

Introduction

Breast cancer is the most common cancer in females in the world. Conventional cancer therapies usually involve chemotherapy and radiotherapy. Chemotherapy is the use of cytotoxic anti-neoplastic drugs to treat several different types of tumors [1]. The emergence of drug resistance has brought major problems in chemotherapy [2]. Drug sensitivity of a tumor is influenced by a variety of aspects, including metabolism of the drug, receptor status of the tumor [3], genomic polymorphism [4] and the effects of drug combinations [4]. Docetaxel (Taxotere) is one of the chemotherapeutic agents that has been mostly used for treatment of breast cancer, alone or in combination with other agents [5].

Until now, few studies have investigated the role of nutrients in improvement of tumors sensitivity to drug resistance [6]. Soy isoflavones belong to a family of nonnutritive compounds modulating a diversity of biological processes linked to carcinogenesis. Genistein, daidzein and glycitein are major isoflavones in soy products that comprise, respectively, 50, 40 and 10% of

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the total soybean isoflavone profile [7]. Many different studies have investigated the effects of soy isoflavones on breast cancer *in vitro* and *in vivo*. Anti-tumor and anti-proliferative effects of isoflavones have been found in several studies [8,9]. Moreover, other useful effects of isoflavones in increasing survival rate and decreasing tumor volume have been reported *in vivo* [10]. While the controversial effects of isoflavones have been investigated in multiple studies, the interference of these nonnutritive agents with chemotherapeutic drugs has been rarely reported so far.

In most experimental studies, where the association of soy isoflavones and breast cancer risk has been investigated, a single component, *e.g.*, genistein or daidzein was employed [9]. But total extracts of isoflavone compared with a single component might exert different *in vivo* effects in metabolism [9]. In addition, synergistic effects among different components of soy isoflavones may occur *in vivo*.

Hence, the aim of this study was to examine the probable effects of soy isoflavone extract on the tumor volume, diet consumption and survival rate of classic chemotherapeutic reagent docetaxel on mouse 4T1 breast tumor model.

Materials and Methods

Cell culture

The 4T1 mouse mammary tumor cell line (estrogen receptor (ER)/progesterone receptor (PR)-negative, National Cell Bank of Iran, Pasteur Institute, Tehran, Iran) was cultured in RPMI 1640 medium (Gibco BRL Inc., Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/ streptomycin, 1 *mM* pyruvate and 2 *mML*-glutamine and maintained in humidified incubator at 37°*C* in 5% CO_2 atmosphere. Tumor cell suspensions greater than 90% viability were prepared from subconfluent cultures with 0.25% trypsin (Invitrogen, CA, USA) and 0.02% EDTA.

Animals

Female BALB/c mice, 6 to 7 weeks old, were purchased from Pasteur Institute of Iran animal facility and kept in a pathogen- free animal facility at Avicenna Research Institute. The animals were housed at standard conditions (24°*C* temperature and 12 *hr* light/ 12 *hr* dark cycles) and allowed to acclimatize for 1 week before starting any experiments. The study was approved by the ethical committees of Shahid Beheshti University of Medical Sciences and Avicenna Research Institute.

In vivo xenograft model of breast cancer

Early passage 4T1 cells (5×10^5) were suspended in 100 μl of phosphate buffered saline (PBS) and injected into the second mammary fat pad of 60 female BALB/c mice. Tumor growth was then assessed twice weekly using vernier calipers, and tumor volumes were calculated by the formula:

V (mm^3)=L (major axis)×W2 (minor axis)/2. At this step, all mice were fed purified American Institute of Nutrition 93 maintenance (AIN 93M) diet, in which soy oil was substituted with corn oil, until treatment time. This diet was used to meet all the requirements of rodents [11]. The food intake and the weight of the mice were measured twice a week. For measurement of the diet consumption, mice were kept in separate cages and allowed to eat and drink ad libitum and the leftover was weighed.

When the tumor volume reached around 50-100 mm^3, the mice were randomly divided to four groups (15 per group): the control group, the dietary soy isoflavones extract group (SIE), the intravenous docetaxel injection group (DOCE) and the combination group of dietary soy isoflavones extract and intravenous docetaxel injection (DOCE+SIE). For groups that were treated with soyisoflavones extract, 100 *mg* soy isoflavones extract was added to AIN 93 *M*. With this supplementation the concentration of isoflavones would be approximately 0.5 *mol/µl* in murine blood [12], which equates to the level of soy consumption among women in their diets [13]. Docetaxel (10 *mg/kg*) was injected intravenously on days 1, 8 and 15 after dividing the mice into groups. The other two groups received sterile PBS on the same days. One week after the last injection, 7 mice from each group were sacrificed and tumors were excised and after fixation in nitrogen, they were stored at -80°*C*. The other 8 mice in each group were monitored for survival rate analysis until they died.

Western blot analysis of MKI67 expression

Sample tissues were homogenized in Ripa lysis buffer (Thermo Scientific, Rockford, IL, USA) on ice using pellet pestle (Sigma-Aldrich, St Louis, MO, USA) for 15 *min* and then centrifuged for 15 *min* at 13,000×*g* at 4 ℃. The supernatants were collected and stored at -80 ℃ until use. BCA assay (Thermo Scientific, Rockford, IL, USA) was applied to measure the protein concentration according to manufacturer's instructions. Sample buffer, with 5% 2-mercaptoethanol, was added to 40 *mg* protein per lane and boiled for 2 *min*. The samples were then separated on 5% SDS-PAGE gel with a 3% stacking gel for 220 *min* at 80 V and then transferred to the nitrocellulose membrane to determine MKI67 expression, as a marker for cell proliferation. The membranes were blocked overnight with 5% skim milk in PBS with 0.05% Tween-20 (PBST). Anti-Mki67 antibody (Abcam, Cambridge, UK, ab-15580) was used to probe the membranes for 2 *hr*. The membranes were subsequently washed with PBST (4×15 *min*) and incubated with 1:8000 dilution of peroxidase-conjugated sheep anti-rabbit antibody (Avicenna Research Institute, Tehran, Iran).

After repeating the washing step, the resulting signal was visualized using ECL Detection kit (ECL Select Amersham, Buckinghamshire, UK) according to the

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manufacturer's instructions. Lysates of Hella cells were used as the positive control for MKI67. Pre-immune rabbit serum was applied instead of primary antibodies in negative control lanes. To ensure equal protein loading, tumor lysates including 40 *mg* protein from each tumor sample were separated on 10% SDS-PAGE gels to determine β-actin expression.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from mouse breast tumor samples using a commercial RNA extraction kit (GeneAll, Biotechnology, Seoul, Korea) based on glass fiber membrane technology, according to the manufacturer's instructions. First, RNA quality was assessed by agarose gel electrophoresis, and then RNA quantity and purity were determined by measuring its absorbance at A260 *nm* and A260/A280 ratio, respectively. The cDNA was synthesized from total RNA by M-MuLV reverse transcriptase and N6 random hexamer. For synthesis of cDNA, 1 *μg* of total RNA, treated with DNase, was heated at 60°*C* for 5 *min* followed by cooling on ice. Master mixture included 4 *μl* of 5× reverse transcriptase buffer, 10 *mM* of each dNTP, 20 pM N6 random hexamer, 1 *μl* RiboLockTMRNase inhibitor, 200 *U* of M-MuLV reverse transcriptase, and DEPC-treated water to a final volume of 20 *μl*. The temperature profile for the cDNA synthesis was 25°*C* for 10 *min*, 42°*C* for 1 *hr* and final heating up to 72°*C* for 10 *min* (all reagents were from Fermentas, Vilnius, Lithuania).

Real time PCR

Analysis of MKI67 gene expression was performed using SYBR green (Takara BIO, Inc., Otsu City, Shiga, Japan) real time PCR. The TATA box binding protein (Tbp), a housekeeping gene, was used as endogenous control.

Two specific primer pairs for MKI67 and Tbp genes were designed using primer 3 software program. The product length of MKI67 using the forward primer 5'-GACAGCTTCCAAAGCTCACC-3' and the reverse primer 5'-TGTGTCCTTAGCTGCCTCCT-3' was 230 *bp*. Amplification of Tbp gene was performed using forward primer 5'-AAGGGAGAATCATGGACCAGA AC-3' and reverse primer 5'-GGTGTTCTGAAT AGG CTGTGGAG-3' which produced a fragment of 149 *bp*. Briefly, 2 *μl* of cDNA were mixed with 1×SYBR Premix EX Taq^{TM}, 0.2 *mM* of each primer and 0.4 *ml* ROX passive reference dye. The amplification was performed in a Rotor-Gene Q real time PCR (QIAGEN, Inc., CA, USA.) as follows: an initial denaturation at 95°*C* for 30 *s*, 40 cycles of 95°*C* for 5 *s*, 60°*C* for 34 *s* and 78°*C* for 5 *s*. For relative quantification of expression of each gene in tumor cells, the following arithmetic formula was used: $2^{-\Delta\Delta CT}$ (Perkin-Elmer Instruction manual, 1997), where the amount of target gene (MKi67) was normalized to Tbp (housekeeping gene) and the relative expression of a gene in tumor was calculated in relation to the mean value of target gene expression in the control group.

Statistical analysis

Data from diet consumption and tumor volume were analyzed using Friedman test followed by Dunn's post hoc test.

MKI67 mRNA expression was analyzed using one-way ANOVA. Also survival analyses were performed using Mantel-Cox method and long-rank test for trend. Differences were considered significant at a p-value of <0.05. All statistical analyses were done using the graphpad prism 5 software program (Graphpad Software. San Diego, CA).

Results

Diet consumption in defined groups

Diet consumption was significantly different among groups (p=0.005) (Figure 1). In this regard, diet consumption in DOCE+SIE was significantly higher than DOCE group.

While diet consumption in SIE group was higher than that in the control group, this difference was not significant.

Survival analysis in defined groups

In survival analysis (Figure 2), the mean survival times in the control, SIE, DOCE and DOCE+SIE groups were 33.3±2.7, 34.7±2.1, 38±1.7 and 40.4±2 days, respectively. While lifespan did not differ significantly between groups (p=0.34), the DOCE+SIE group had the longest lifespan among the groups.

Breast tumor volume

Figure 3 shows that there were no significant differences between tumor volumes of the groups. A slight decrease in tumor volume was seen in DOCE+SIE group compared to DOCE group. Soy isoflavone ex-

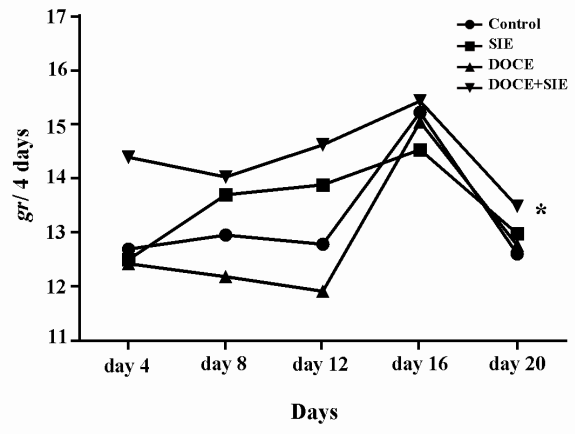

Figure 1. The rate of diet consumption every 4 days in different mouse groups (15 per group). Significant difference in consumption rate between DOCE+SIE and DOCE groups was detected. Control group received AIN 93 *M* diet, the dietary soy isoflavone extract (SIE) group received AIN 93M diet+100 *mg* SIE, the intravenous docetaxel injection (DOCE) group received 10 *mg/kg* DOCE and the combination group received soy isoflavone extract and intravenous docetaxel injection (DOCE+SIE) *p<0.01.

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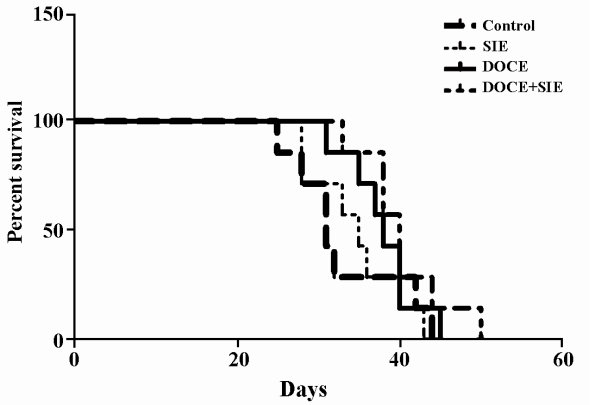

Figure 2. Survival curves of 4T1 induced breast cancer mouse models in different treatment groups (8 per group). The mean survival times in the control, SIE, DOCE and DOCE+SIE groups were 33.3± 2.7, 34.7±2.1, 38±1.7 and 40.4±2 days, respectively. No significant differences were detected between groups.

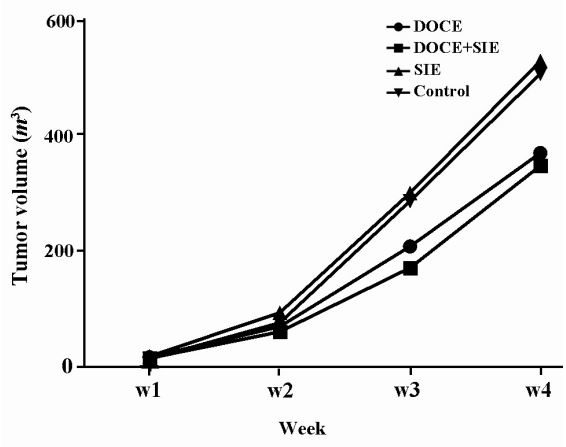

Figure 3. Effect of soy isoflavone extract on tumor volume of 4T1 mice mammary fat pad tumors (15 per group). The means of tumor volume in control, SIE, DOCE and DOCE+SIE groups were 221± 111, 235±114, 166±79 and 148±74 mm^3, respectively. No significant differences were detected between groups. Control group received AIN 93M diet, the dietary soy isoflavones extract (SIE) group received AIN 93 *M* diet+100 *mg* SIE, the intravenous docetaxel injection (DOCE) group received 10 *mg/kg* DOCE and the combination group received soy isoflavone extract and intravenous docetaxel injection (DOCE+SIE).

tract exerted no effect on tumor volume in SIE group compared to the control group. The means of tumor volume in control, SIE, DOCE and DOCE+SIE groups were 221±111, 235±114, 166±79 and 148±74 mm^3, respectively.

***Gene and protein* expressions of MKI67**

No significant differences in MKi67gene expressions were observed among the groups. However, it seems that SIE may induce MKI67 mRNA expression and thereby cell proliferation (Figure 4). In western blot analysis two bands of 345 and 395 *kDa*, related to MKI67 protein were detected. The lowest expression of MKI67 protein was seen in DOCE+SIE group and

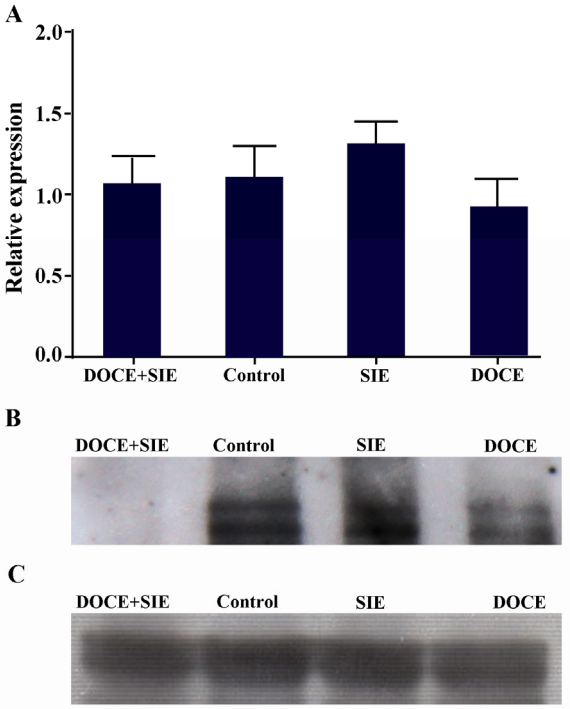

Figure 4. MKI67 mRNA and protein expressions in different groups. The control, the dietary Soy Isoflavones Extract (SIE), the intravenous docetaxel injection (DOCE) and the combination group of dietary soy isoflavone extract and intravenous docetaxel injection (DOCE+SIE) groups are shown. A) Relative expression of MKI67 gene in different groups; B) Western blot analysis of MKI67; C) β-actin. No significant differences between groups were detected.

an increase in its expression in DOCE was observed in SIE and the control groups, respectively.

Discussion

Chemotherapy is the most common therapy among cancer patients [14]. However, chemotherapy has been known to induce toxicity and drug resistance in cancer cells, causing treatment failure [5]. Using a nontoxic agent with a different mechanism of action in combination with chemotherapy agents is imperative in order to promote new regimens that can augment the therapeutic efficacy of chemotherapeutic agents and reduce their toxicities [15]. Soy isoflavones, nontoxic flavonoid compounds, are dietary agents that have been reported to prevent cancer and may increase the efficacy of cancer treatment by modifying the activity of key survival pathways and cell proliferation [16]. Different patterns of anti-tumor activity have been proposed for single isoflavones compared to their combinations [8]. In this regard, the present study was conducted to compare the anti-tumor effects of soy isoflavone extract alone and in combination with docetaxel on 4T1 cell-line induced murine breast cancer model.

Appetite and immune status are significantly decreased in cancer patients [17]. Pro-inflammatory cytokines from tumors are associated with fatigue, anorex-

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ia, cachexia pain and resistance to treatment that reduce the quality of life for cancer patients which is further significantly reduced during chemotherapy due to a wide range of side effects such as decrease of appetite and vomiting [18]. Our results showed that diet consumption in DOCE+SIE group was significantly higher than that in DOCE group. The results are in agreement with those of Rohr *et al's* [17] who found that daily consumption of fermented soy significantly increased appetite and decreased cytokine TNF-α level in cancer patient sera during chemotherapy compared to placebo group. Reduction of cytokines such as interleukin 6 (IL-6) due to soy isoflavone consumption may be another possible explanation [19].

Several studies have shown that consumption of soy products improve survival rate and decrease risk of death and recurrence of cancer among women with breast cancer [20-22]. The improvement in nutritional status can improve survival. The beneficial effects of soy consumption on the cardiovascular system, bone health and degenerative arthritis has been reported in the literature [17,22].

However, higher but not significant diet consumption was observed when SIE was used alone in comparison to the control group. The toxic side effects of docetaxel may cause more suppressive effect on diet consumption and therefore SIE may be more effective on diet consumption in treated groups in comparison with untreated groups.

Previous report has showed that treatment of NB4 and HL-60 xenograft mice with soy isoflavone genistein combined with chemotherapy agent significantly increased survival time in comparison to untreated control group. In this study, no significant differences were found in survival times among the four groups. The non-significant improvement of survival time that was found in our study may be due to several factors such as use of low-dose docetaxel and more aggressive and metastatic character of 4T1 breast cancer than other kinds of breast cancer [6].

Although epidemiologic study has reported that soy protection against breast cancer is independent at the Estrogen Receptor (ER) status, irrespective of whether a breast cancer is ER-negative or ER-positive [20], inconsistent data exist on the effects of pure phytoestrogens or soy products on breast tumor progression. While *in vitro* and *in vivo* anti-tumor activities of genistein have been reported in different types of breast cancer [8,18], some studies have shown the stimulating effect of genistein on the tumor growth of ER-positive breast cancer [23]. Promoting and inhibitory effects of daidzin on ER-positive and ER-negative breast cancer cells have been found in several studies [18,24]. This discrepancy may be due to the use of specific isoflavones alone instead of combination of soy isoflavones. In support of this theory, Kim *et al* [9] compared the effect of diets containing soy extract and genistein on the growth of implanted estrogen-independent human breast cancer cells (MDA-MB-231) in female BALB/c mice. It was reported that the effect of soy extract in inhibiting tumor growth was more potent than genistein. This result may be due to the synergistic effect of different bioactive components in the soy extract.

It was reported that tumor growth of estrogen-unresponsive (MDA-MB-231) and estrogen-dependent (MCF-7) human breast cancer xenografts in athymic mice was not affected by phytoestrogens-containing soy extract. Similarly, Martinez-Montemayor *et al* [18] stated that combined soy isoflavones had no effect on primary tumor growth in a mouse breast cancer model. Consistent with these reports, our results showed that tumor (volume) was not affected by soy isoflavone extract and there were no significant differences between the soy isoflavone and the control groups. Though the tumor volume in DOCE+SIE group was lower than that in DOCE group, this difference was not significant. These results could be in line with our findings in western blot, which showed that in mice treated with DOCE+SIE, expression of MKI67 was lower than that in DOCE group and also there were no differences in MKI67protein expression between SIE and control groups. However, the basic mRNA expression level of MKI67 was not significantly different among groups. Our results confirm the findings of previous study [24] that there were no significant differences in the expression of MKI67 in estrogen-dependent mice, received 25 *g/kg* soy isoflavone extract, in comparison with the control. It is suggested that the discrepancy between MKI67 mRNA levels and protein levels in DOCE+SIE group could be due to non translational regulation and intracellular storage of mRNA which is affected by such interaction [25].

Conclusion

In conclusion, our results showed that combined therapy with docetaxel had synergistic effects on diet consumption.

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