Original article

Combination of arabinogalactan and curcumin induces apoptosis in breast cancer cells in vitro and inhibits tumor growth via overexpression of p53 level in vivo

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Purpose: Increased mortality associated with breast cancer in women has spurred the studies to develop new drugs. Arabinogalactan (AG) and curcumin (Cur) are two natural products broadly explored in cancer therapy. Our major goal in the current study was to assess anticancer properties of combination these reagents in vitro on human breast cancer cells and in vivo utilizing animal model of breast cancer.

Experimental design: We evaluated cell proliferation, apoptosis, cell cycle, and protein expression in vitro on MDA-MB-231 human breast cancer cells. For in vivo studies, murine breast cancer cells were implanted into BALB/c mice. Thereafter, volume of the developing tumor was calculated and expression of Ki67 and p53 proteins was evaluated to analyze cell proliferation and apoptosis.

Results: Combination of AG and Cur significantly decreased cell growth in human breast cancer cells without any significant effect on normal cell growth. This combination could increase cell population in sub-G1 phase, which was indicative of apoptosis. Western blotting showed that the combination of AG and Cur significantly increased Bax/Bcl2 ratio as well as cleaved-caspase3 level in MDA-MB-231 cells. Combination of AG and Cur promoted apoptosis by increasing ROS level, changing mitochondrial membrane and reduction of glutathione. In addition, in vivo studies in mouse showed that this combination could inhibit the progression of breast tumors through over-expression of p53 and reduction of Ki67 levels.

Conclusion: Our findings suggest that the combination of AG and Cur is of great potential to induce apoptosis in breast cancer cells in vitro and in vivo.

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1. Introduction

Breast cancer is the most common malignancy and the second important cause of cancer death among woman after lung cancer [1]. The high morbidity and mortality rates are mainly due to metastasis, which generally comprises the following stages: invasion, intravasation, circulation, extravasation, and colonization in distant organs (e.g. lung, bone, and liver) [2]. Conventional cancer therapy methods including surgery, radiotherapy, and chemotherapy can offer major curative effects against the localized tumors. However, these treatments show poor effects on suppressing the invasion and metastasis of breast cancer, which can lead to reappearance of the tumor [3]. On the basis of numerous epidemiological findings that diet plays a key role in cancer prevention, recent efforts have been concentrated on traditional medicine (e.g. spices used in cooking) as a powerful source of phytochemicals with proved potency to be used in the treatment of chronic diseases, particularly cancer [4,5]. Larch Arabinogalactan (AG) is a polysaccharide extracted from the wood of Larch tree (Larix genus) [6]. AG may be a valuable agent for cancer therapies due to its potential to reduce cell proliferation in cervix cancer [7] and to block metastasis of liver tumor cells [8]. Curcumin (Cur) is another well-studied chemo-preventive component, which is the major yellow pigment in turmeric and has been demonstrated to exert anti-tumor effects on different cancer cell lines and animal models. Cur has been shown to induce cell cycle arrest and thus inhibit the proliferation and survival of cancer cells [9]. Moreover, it is a potent inducer of apoptosis in many cancer cells in vitro and in vivo [10]. Dysfunction of apoptosis, which is a physiologic process of programmed cell death, has been considered as a feature of most types of cancers. Therefore, the current anticancer therapies mostly focus on inducing apoptosis in cancer cells as an effective treatment. Mitochondria have been
attributed a key role (perhaps even a central role) in the apoptotic process through the intrinsic pathway of this phenomenon [11]. Oxidative stress with production of reactive oxygen species (ROS), can lead to disturbance in mitochondrial potential and may result in its permeability, which is regulated by members of Bcl-2 family. Glutathione (GSH) is the major non-protein antioxidant in the cell and supplies electrons for glutathione peroxidase enzyme, which helps to eliminate free radicals such as ROS from the cells. GSH has been shown to be a highly essential factor for cell proliferation and apoptosis. Studies revealed that anticancer drugs can lead to reduction in the GSH level of cancer cells [12,13]. Recently it is reported that AG as a galactose polysaccharide can play a significant role in increasing absorption of drugs such as Cur via galectin-mediated absorption. Specifically they have demonstrated that AG-covered Cur nanoparticles could intensify bioenhancement of oral Cur due to augmenting its gastric maintenance [14]. So far to our knowledge the combination of AG and Cur was not explored on any type of cancer. Therefore, in our study, we explored the effect of combination of two natural agents AG and Cur on breast cancer cell growth in vitro and in vivo and subsequently investigated the mechanism of action of the mentioned compounds.

2. Materials and methods

2.1. Materials and chemicals

Cur was supplied from Serva Co. (USA). AG, 2, 7- dichlorodihydro fluorescein diacetate (DCFDA), Rhodamin123, 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Acridine Orange (AO), Ethidium Bromide (EB), hematoxylin and eosin dye as well as RNase A were purchased from Sigma Aldrich Co. (UK). RPMI 1640 media, Fetal Bovine Serum (FBS) and Trypsin/EDTA were obtained from Gibco Co. (USA). Secondary antibodies were supplied from Cell Signaling Co. (UK). Polyvinylidene fluoride (PVDF) and ECL Kit were provided from Amersham Bioscience Co. (USA). Ki67 and p53 primary antibodies, avidin-biotin-peroxidase and DAB reagent were provided by Abcam Co. (UK).

2.2. Cell culture

MDA-MB-231 human breast cancer cell line, 4T1 mouse breast cancer cell line and human umbilical vein endothelial cell line (HUVEC) were obtained from NCBI (national cell bank of Iran). The cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% antibiotic/antimitotic solution and 0.2% NaHCO3. All cells were cultured in a humidified incubator at 37 °C containing 5% CO2 [15].

2.3. Cell growth assay

Analysis of the anti-proliferative effects of AG and Cur on breast cancer cells and HUVEC cells was conducted by MTT assay. The cells were seeded in 96-well plates at a density of 104 cell/well in a final volume of 200 μl. Then cells were treated with adequate concentrations of AG or Cur after an overnight incubation while DMSO (the solvent) was used as control. After 24 h of incubation, 20 μl of MTT (5 mg/ml) was added to each well. The cells were incubated at 37 °C for 3 h. Thereafter, the medium was taken away and the insoluble formazan crystals were dissolved in 200 μl of DMSO. The absorbance was measured at 490nm by Microplate Reader (BioTek, UK). The results were expressed as the percentage of cell growth relative to the control [16].

2.4. Acridine orange (AO) and ethidium bromide (EB) double staining

After treatment with the combination of AG (10 mg/ml) and Cur (75 μM) for 24 h, the cells were stained with AO/EB fluorescent dyes for 5 min, then detached and washed by cold PBS, and finally stained with a mixture of AO (100 μg/ml) and EB (100 μg/ml) at room temperature for 5 min. The stained cells were scrutinized by means of a fluorescence microscope (Zeiss, Germany) at 100× magnification. In each experiment, more than 300 cells per sample were counted [17].

2.5. Nitrite (NO2−) detection

For evaluation of the amount of NO release, we utilized Griess reagent under acidic conditions to record the accumulated nitrite (NO2−), which is a stable breakdown product of NO. During the assay, medium aliquots were mixed with equal volumes of Griess reagent and incubated at room temperature for 15 min. To analyze the azo dye production, a spectrophotometer with absorbance set at 490 nm was used. Sodium nitrite was used as a standard [18].

2.6. Detection of intracellular reactive oxygen species (ROS) levels

Intracellular ROS were spotted using an oxidation-sensitive fluorescent probe dye, 2, 7- dichlorodihydro fluorescein diacetate (DCFDA). Concisely, 1 × 106 cells were incubated in a 60 mm culture dish with combination of AG (10 mg/ml) and Cur (75 μM) for 24 h. The cells were then washed in PBS and incubated for 30 min at 37 °C with PBS containing 20 μM DCFDA. The samples were analyzed using a FACScan flow cytometer [19].

2.7. Measurement of glutathione (GSH) level

The concentration of glutathione (GSH) was determined in MDA-MB-231 cells using DTNB method at 412 nm [20].

2.8. Measurement of the mitochondrial membrane potential (MMP)

MMP was assessed by applying rhodamine 123 as a fluorescent dye (Ex/Em = 485 nm/535 nm). After treatment, the cells were trypsinized and suspended in 1 ml PBS. Next, 3 μl of 1 mg/ml rhodamine 123 was added to cell suspension and incubated for 10 min at 25 °C. The cells were then washed twice with PBS and observed with a fluorescence microscope (Zeiss, Germany) at 200× magnification. Also, MMP was measured using a FACStar flow cytometer as previously described [21]. In brief, 1 × 106 cells in a 60 mm culture dish were incubated with combination of AG (10 mg/ml) and Cur (75 μM) for 24 h. The cells were washed twice with PBS and incubated with rhodamine 123 (0.1 μg/ml) at 37 °C for 30 min. The absence of rhodamine 123 from cells showed the loss of MMP in the cells [22].

2.9. Cell cycle analysis

The cells were fixed, stained with Propidium iodide (PI) dye and analyzed by flow cytometer. Since PI dye binds to both DNA and RNA, RNase (ribonuclease) was used to digest RNA to reduce background staining. The resulting DNA was quantified by FlowJo software for detecting the proportion of cells in G1, S, and G2 phases of the cell cycle [23].

2.10. Western blotting

After the specified treatment, cells were collected and the protein extraction was conducted. At first, the protein concentration was calculated by Bradford’s method [24]. Equivalent amounts
Fig. 1. Effect of arabinogalactan (AG) and curcumin (Cur) on cell growth after 24 h using MTT assay. (A) Effect of different AG concentrations (0.25, 0.5, 1, 2, 4, 6, 8, 10 and 12 mg/ml) on cancer cell growth. (B) Effect of different Cur concentrations (1, 5, 10, 25, 50, 75, 100 and 200 μM) on cancer cell growth. (C) Effect of the combination of AG (0.5 and 10 mg/ml) and Cur (10 and 75 μM) on cancer cell growth. (D) Effect of AG, Cur and the combination of their effective doses on the growth of normal HUVEC cell line. *** p < 0.001, ** p < 0.01 compared to the control, # p < 0.05 compared to AG (10 mg/ml), + p < 0.05 compared to Cur (75 μM).
Fig. 2. (A) AO/EB staining of MDA-MB-231 cells after 24 h treatment with AG (10 mg/ml), Cur (75 μM) and their combination. Green cells indicate live cells (shown with green arrows), yellow cells indicate apoptotic cells (shown with yellow arrows) and red cells indicate necrotic cells (shown with red arrows) (magnification at 100×). (B) Percentage of apoptotic cells, (C) Percentage of necrotic cells. *** p < 0.001 compared to control, ### p < 0.001 compared to AG (10 mg/ml), ** p < 0.01 compared to Cur (75 μM).
of protein were boiled for 5 min and detached by SDS-PAGE and were then transferred onto a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was then blocked with 5% nonfat dry milk in Tris-Buffered-Saline with Tween (TBST) for 1 h at room temperature and incubated with suitable primary antibodies overnight at 4 °C. Subsequently, the membrane was washed with TBST and incubated with appropriate secondary antibody for 1 h at room temperature. After three washing steps with TBST, the proteins were observed employing the electrochemiluminescence (ECL) reagent. Analysis of the integrated density of the resultant protein bands was performed by Image J software [25].

2.11. Animals and treatment

Female inbred BALB/c mice at 6–8 weeks of age were purchased from Pasteur Institute (Tehran, Iran). They were kept at 22–24 °C with a 12 h light/dark cycle in an utterly designed pathogen-free isolation facility and were adapted at one week prior to experimentation. The University of Tehran animal ethics committee approved all procedures. A total of 1 × 10⁶ 4T1 cells/200 µl in logarithmic growth phase were subcutaneously (s.c.) injected into the right flank of BALB/c mice. Once the tumor mass was established on day 12, the mice were randomly assigned to various treatment groups (7 mice per group). The mice were orally administrated with AG (50 mg/kg/day), Cur (100 mg/kg/day), combination of AG and Cur, and PBS (5 ml/kg/day as control) for 32 days. Tumor volume (mm³) was measured twice a week using a digital Vernier Caliper (Mitutoyo) until the mice were sacrificed on day 32 post injection. The tumor volume was calculated by the following formula: Length × width² × π/6 [26,27].

2.12. Assessment of animal health

Body weight of mice was daily controlled throughout the treatment period. The mice were euthanized by CO₂ injection into the chamber and necropsies were then performed. Vital organs such as kidney, spleen, liver as well as the developed tumors were excised and weighed. Organ weights were expressed as a percentage of body weight [27].

2.13. Histological analysis

The excised tumor was fixed in 10% formalin, dehydrated in graded alcohol solutions, embedded in Paraplast plus® (Oxford Labware, Memphis, TN, USA) and cut into 5 μm thick serial sections. The histological hematoxylin and eosin staining (H&E) method was used for morphological and pathological evaluation of the tumor samples. For further qualitative pathological studies, each slide was photographed using a camera coupled to microscope (Olympus-BX51BX51, America, Center Valley PA) at 400× magnification [28].

2.14. Immunohistochemical staining

Paraffin-embedded tumor sections were deparaffinized using dimethylbenzene after 20 min and rehydration was performed in graded concentrations of ethanol (100, 90, 80, and 70%). Then, the immunohistochemical process was done on the sections as formerly described [29]. Briefly, after blocking endogenous peroxidase using 3% hydrogen peroxidase, the sections were treated with primary mouse anti-human monoclonal Ki67 and p53 antibodies (1:100), which were diluted in phosphate-buffered saline containing 0.1% Tween-20 (PBST) and 5% bovine serum albumin. After an overnight incubation at 4 °C, the sections were treated with the secondary antibody (1:100), avidin-biotin-peroxidase and DAB. At the end, hematoxylin dye was used for counterstaining and the sections were visualized by microscopy (Olympus-BX51BX51, America, Center Valley PA).

2.15. Data analysis

All data were represented as mean ± SEM. Comparison between the groups was made by one-way analysis of variance (ANOVA) followed by a specific post hoc test to analyze the differences. The statistical significance was achieved with p values less than 0.05.

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**Fig. 3.** Effect of AG (10 mg/ml) and Cur (75 μM) on NO production in MDA-MB-231 cell line after 24 h. The results are expressed as mean ± SEM (N=3), ***p < 0.001 compared to the control, ### p < 0.01 compared to AG (10 mg/ml), ++ p < 0.05 compared to Cur (75 μM).
3. Results

3.1. Combination of AG and Cur decreased cell growth in MDA-MB-231 cells without any significant effect on growth of HUVEC normal cell line

We applied MTT assay to inspect the effect of AG and Cur on the growth of MDA-MB-231 breast cancer cell line. The cells were treated with different concentrations of either AG or Cur for 24 h (Fig. 1A and B). We observed that AG or Cur can reduce cell growth in a dose-dependent manner. The IC$_{50}$ for AG was nearly 10 mg/ml and that of Cur was approximately 75 μM in 24 h. As a result, we chose 0.5 mg/ml concentration of AG as the non-effective dose and 10 mg/ml as the effective one. We demonstrated that the non-effective and effective doses for Cur were 10 mg/ml and 75 μM, respectively. Our data showed that the combination of effective doses of AG and Cur considerably restrained the cell growth at 24 h incubation. The combination of AG (10 mg/ml) and Cur (75 μM) significantly decreased the cell growth to 66 ± 3.4%, 23 ± 1.8% and 21% ± 2.2% compared to the control (DMSO 1%), AG (10 mg/ml) and Cur (75 μM) groups, respectively (Fig. 1C). Interestingly, the combination of effective doses of AG and Cur had no significant effect on the growth of HUVEC human normal cells (Fig. 1D). Therefore, the combination of effective doses of these compounds was employed for the rest of the study in all the experiments.

3.2. AO/EB double staining analysis showed increased apoptotic cells in the combination of AG and Cur

Fluorescent staining of cells with AO/EB showed that the viable cells with normal DNA have green nuclei, while the apoptotic and necrotic cells possess yellow and red nuclei (Fig. 2A). It was demonstrated that the combination of AG (10 mg/ml) and Cur (75 μM) increased the number of apoptotic cells in comparison with the cells treated with AG (10 mg/ml) or Cur (75 μM) alone (Fig. 2B). It was also observed that the number of necrotic cells did not increase significantly in this group compared to control group (Fig. 2C).

3.3. Combination of AG and Cur increased Nitric Oxide (NO$_2^-$) production in MDA-MB-231 cells

The MDA-MB-231 cells were treated with AG (10 mg/ml), Cur (75 μM) and their combination for 24 h. Our experiments revealed that nitrite (NO$_2^-$) production was increased in the presence of either AG (10 mg/ml) or Cur (75 μM). Moreover, the combination of AG (10 mg/ml) and Cur (75 μM) significantly increased nitrite (NO$_2^-$) production in comparison with the use of the aforementioned compounds alone [1.6 ± 0.25 fold compared to the control, 1.2 ± 0.18 fold compared to AG (10 mg/ml) and 1.2 ± 0.13 fold compared to Cur (75 μM) groups (Fig. 3)].

3.4. Combination of AG and Cur augmented intracellular ROS level in MDA-MB-231 cells

Flow cytometry analysis demonstrated that the treatment of cells with either AG (10 mg/ml) or Cur (75 μM) could raise the intracellular ROS levels. The combination of AG and Cur generated even more ROS in comparison with AG or Cur alone [(2.2 ± 0.3 fold compared to the control, 1.2 ± 0.16 fold compared to AG (10 mg/ml) and 1.2 ± 1.4 fold compared to Cur (75 μM) groups) (Fig. 4)].

3.5. The combination of AG and Cur decreased intracellular GSH level in breast cancer cells

Decreased intracellular glutathione (GSH) levels would be indicative of apoptosis. The combination of AG (10 mg/ml) and Cur (75 μM) resulted in a dramatic decrease of GSH level in comparison with either AG or Cur groups. In the combination group, GSH level was decreased by 45%, 20% and 23% compared to the control, AG (10 mg/ml) and Cur (75 μM) groups, respectively (Table 1).

3.6. Combination of AG and Cur decreased MMP in MDA-MB-231 cells

We assessed the effect of combination of AG (10 mg/ml) and Cur (75 μM) on MMP as a major mitochondrial parameter that controls the cellular processes. In fact, MMP reduction would lead to the initiation of apoptosis and the absence of rhodamine 123 from the cells indicates the loss of MMP. Treatment of cells with either AG

![Fig. 4](image_url) Effect of AG (10 mg/ml) and Cur (75 μM) on ROS levels in MDA-MB-231 cell line after 24 h. The results are expressed as mean ± SEM (N=3), *** p < 0.001 compared to control. ## p < 0.01 compared to AG (10 mg/ml), ** p < 0.05 compared to Cur (75 μM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH (μmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td>AG 10 mg/ml</td>
<td>0.39 ± 0.02***</td>
</tr>
<tr>
<td>Cur 75 μM</td>
<td>0.38 ± 0.02***</td>
</tr>
<tr>
<td>AG 10 mg/ml + Cur 75 μM</td>
<td>0.3 ± 0.02**</td>
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Table 1 GSH levels in treated MDA-MB-231 cells were measured in 412 nm wavelength. The results are expressed as mean ± SEM (N=3), *** p < 0.001 compared to control. # p < 0.001 compared to AG (10 mg/ml), * p < 0.05 compared to Cur (75 μM).
(10 mg/ml) or Cur (75 μM) decreased MMP in MDA-MB-231 cells; however, the combination of AG and Cur caused a sharp decline in MMP in comparison with AG or Cur alone (Fig. 5).

3.7. Combination of AG and Cur increased sub-G1 phase in MDA-MB-231 cells

Flow cytometry analysis was conducted to identify the distribution of cells during cell cycle phases. As shown in Fig. 6, a sub-G1 phase indicative of apoptotic cells appeared in the cell cycle of cells. After exposure to 10 mg/ml of AG, the population of apoptotic cells reached 18.5% compared to 0.8% in control group. After treatment with Cur (75 μM) the percentage of apoptotic cells elevated to 31% compared to control group. However, the combination of AG (10 mg/ml) and Cur (75 μM) caused a significant increase in the sub-G1 population to 50.1% compared to either AG or Cur alone.

3.8. Combination of AG and Cur changed the expression level of apoptotic-related proteins

Western blot was performed to assess the expression of Bcl-2, Bax, and caspase3. Our results demonstrated that the combination of AG and Cur significantly increased Bax/Bcl-2 ratio in comparison with AG or Cur alone (p < 0.001). Furthermore, the combination of AG and Cur significantly increased the level of cleaved caspase3 in comparison with either AG or Cur alone (p < 0.001 relative to AG and p < 0.01 relative to Cur) (Fig. 7).

3.9. Tumor growth was decreased following combined treatment in vivo

To determine in vivo safety and efficiency of the combined treatment, female BALB/c mice were injected with murine breast cancer 4T1 cells and treated with AG (50 mg/kg), Cur (100 mg/kg) and a combination of these two compounds for a period of 32 days. The selected concentrations were chosen based on previous

![Fig. 5. Effect of AG (10 mg/ml) and Cur (75 μM) on MMP in MDA-MB-231 cells after 24 h. (A) MMP loss in the cells was observed with fluorescent microscope. Cells with normal MMP are shown with red arrows and cells with lost MMP are indicated with purple arrows. (B) Graphs indicate the percentage of MMP loss in the treated cells and control group. The results are expressed as mean ± SEM (N = 3), ** p < 0.001 and *** p < 0.01 compared to the control, ### p < 0.01 compared to AG (10 mg/ml), and * p < 0.01 compared to Cur (75 μM).](image-url)
Our in vivo results demonstrated that mean tumor volume in AG treated mice was 26% smaller than the control group, while no significant change was observed in the Cur treated group. Interestingly, mean tumor volume in the mice treated with the combination of AG and Cur significantly decreased to 40%, 26% and 32% compared to control, AG, and Cur, respectively ($p < 0.01$ relative to AG and $p < 0.001$ relative to Cur) (Fig. 8A). Furthermore, mean tumor weight in the combination group was 68% less than the control group. Amazingly, the combination of AG and Cur significantly decreased tumor weight to 30% and 58% compared to AG or Cur treated mice, respectively ($p < 0.01$ relative to AG and $p < 0.001$ relative to Cur) (Fig. 8B).

3.10. The combination of AG and Cur decreased the nucleus to cytoplasm ratio

Histological examination was done through H&E staining of tumor sections. Our results indicated that the tumor tissue derived from control mice has a disordered arrangement of cancer cells and a high cell density. The cells were ovoid or round and showed a high nucleus to cytoplasm ratio. On the contrary, in tumor tissues derived from animals treated with the combination of AG and Cur, the ratio of nucleus to cytoplasm was reduced compared to control group and the nuclei of the majority of cells were polygonal and lightly stained. However, in tumor tissues derived from animals treated with either AG or Cur, the nucleus to cytoplasm ratio showed little reduction relative to control (Fig. 9A).

Fig. 6. Effect of AG (10 mg/ml) and Cur (75 μM) on cell cycle. The histograms show the effect of AG or Cur or their combination on cell cycle distribution of MDA-MB-231 cells after 24 h. The sub-G1 percentage indicates the population of apoptotic cells.
Fig. 7. Western blotting analysis of apoptotic proteins in MDA-MB-231 cells. A) Effect of AG (10 mg/ml) and Cur (75 μM) on the expression of Bax, Bcl-2, and cleaved caspase3 levels. The density of: B) Bax/Bcl2 ratio and C) cleaved caspase3 bands was measured, and their ratio to β-actin was calculated. The mean of three independent experiments is shown. *** p < 0.001 and ** p < 0.01 compared to the control, ### p < 0.001 compared to the AG (10 mg/ml), +++ p < 0.001 and ++ p < 0.01 compared to the Cur (75 μM) group.
3.11. The combination of AG and Cur decreased Ki67 and increased p53 protein levels in vivo

Immunohistochemistry analysis demonstrated a significant downregulation of Ki67 expression in tumor tissues following treatment with the combination of AG and Cur in comparison with each agent alone (Fig. 9B). Moreover, the assessment of p53 expression indicated the upregulation of this apoptosis inductive protein in the combination group compared to AG or Cur alone (Fig. 9C).

3.12. A combination of AG and Cur had no side effects on vital organs

Chemotherapy reagents have many side effects on vital organs such as liver, spleen, and kidney. Moreover, their consumption results in severe weight loss in treated patients in most cases. Therefore, we assessed the mentioned parameters in the BALB-c mouse model bearing 4T1 breast cancer. Our data showed that the body weight in mice treated with the combination of AG and Cur was not different from the control mice treated with PBS. Besides, the weight of liver, kidney, and spleen was normal and no obvious change was observed in the combination group relative to control (Table 2).
4. Discussion

The therapeutic methods based on drug combinations concentrate on enhancing clinical responses while minimizing the side effects as well as drug resistance rate. The benefit of combining multiple compounds arises from the fact that each agent can have a single target or mechanism of action or those different agents may share the same target or mechanism of action against cancer cells. Thus, the combination treatment could either develop the number of targets and/or the mechanisms of action or increase the effects on the same target; therefore, it would result in less drug concentration required for an efficient therapy [32].

Cur is a biphenyl compound obtained from C. longa and AG is an herbal medicine extracted from the wood of larch. Both are widely used in traditional medicine for treating various diseases [8,9,33]. Cur and AG have been individually reported to have anti-tumorigenic effects on several types of human cancers [8,9]. These findings are important because the problem of toxicity and drug resistance related to cancer chemotherapeutic agents has created a challenge in the field of cancer studies and the need for an alternative therapy is clearly felt [34].

Impairment of cell proliferation and apoptosis is an obvious hallmark of cancer cells. To grow in a dysregulated manner, cancer cells become resistant to anti-proliferative signals and propagate in the absence of exogenous mitogenic growth factors. Moreover, the number of cells is controlled by apoptosis, the programmed cell death. However, cancer cells can exploit several mechanisms to circumvent this regulatory signal and thus do not experience apoptosis under suitable conditions [35]. Consistent with previous studies, our results demonstrated that either Cur or AG alone could inhibit the growth of breast cancer cells in a dose dependent manner in vitro. Interestingly, we showed that the combination of AG and Cur decreased breast cancer cell growth more strongly than either agent alone. Reactive oxygen species (ROS) are a group of highly reactive chemicals under severe control of intracellular antioxidants. The balance of oxidation-antioxidation is vital for preserving normal cell functions and any imbalance in this respect could result in a wide range of diseases, including cancer. There are many evidences indicating that ROS can act as cancer suppressors. Therefore, induction of ROS production is a mechanism shared by all non-surgical therapeutic tactics for cancers, including chemotherapy, radiotherapy and photodynamic therapy. [36]. Our findings highlighted that the combination of AG and Cur increased ROS levels in cancer cells to a higher extent than when either drug was employed alone. When present in very high concentration, ROS can damage important cellular structures, giving rise to senescence and fatal lesions in cancer cells [13]. The glutathione (GSH) content of cancer cells is particularly relevant to the regulation of DNA synthesis, growth, and multidrug resistance. Compared with normal tissues, the resistance is associated with higher GSH levels in most cases in malignant tumors [37]. Our results demonstrated that the combination of AG and Cur decreased GSH level to a greater extent than when either drug was used alone. Thus, our approach of using this combination can be a possible strategy for cancer therapy by inducing GSH depletion in these cells [37,38]. Previously, it was shown that apoptosis in most cells is accompanied with an augmentation in the population of cells in sub-G1 phase of cell cycle [17]. Our results proved that the apoptosis induced by the combination of AG and Cur was mostly associated with an increase in the number of cells in sub-G1 phase. Alterations in mitochondrial membrane potential (MMP) is one of the early characteristics of intrinsic apoptotic pathway [11,39]. It was shown in this study that the combination of AG and Cur significantly reduced the MMP compared to usage of each compound alone. Reduced mitochondrial membrane potential in the presence of these compounds can lead to the initiation of
apoptosis in our breast cancer cell line. The intrinsic pathway of apoptosis is regulated by the proteins of Bcl2 family, which can be categorized into pro-apoptotic (such as Bax) and anti-apoptotic (such as Bcl-2) proteins. The balance and location of these two types of proteins influences the cell fate [40]. In many cancers, the anti-apoptotic members of Bcl-2 family are commonly upregulated while the pro-apoptotic members are downregulated [11,41]. Our study showed that the Bax/Bcl-2 ratio in MDA-MB-231 cells was also significantly increased after treatment with the combination of AG and Cur. Increased Bax/Bcl-2 ratio can result in mitochondrial dysfunction, release of some apoptotic factors and eventual activation of caspase3. [40]. In accordance with this fact, our data demonstrated that the enhancement of caspase3 activation was stronger in the combination of AG and Cur group compared to each reagent alone.

Cur has been reported to be relatively safe and well tolerated by animals and humans even at high doses [27]. However, its poor absorption by intestinal tract and rapid removal through chemical modifications in liver would limit its availability to the epithelial cells of the intestine and colon [42,43]. Therefore, to further support our in vitro findings, we extended our experiments to mouse models of breast tumor. Our data depicted that the combination of AG and Cur significantly decreased tumor volume and weight compared to either AG or Cur alone. Ki67 is a nuclear protein correlated with cellular proliferation, which is expressed in all phases of the cell cycle and its evaluation shows the growth fraction of neoplastic cell populations [44]. Our results from immunostaining of Ki67 indicated that tumor cell proliferation was significantly diminished in the mice treated with the combination of these two agents relative to each compound alone. This reduction in cell proliferation was accompanied with a dramatic expression of p53 protein, indicative of cell apoptosis, in the combination treatment. Interestingly, no side effect was observed in vital organs of the recipient mice in the combination group, which may raise it as a candidate for safe treatment of cancer.

5. Conclusion

In conclusion, our results indicated that the treatment of breast cancer cells with the combination of AG and Cur can be more effective in inhibiting in vitro and in vivo cancer cell growth in comparison to the treatment with AG or Cur alone. The ability of combination of AG and Cur to stimulate apoptosis in breast cancer cells points to the possibility of developing the combination therapy as a potential cancer chemoprevention approach.

Conflict of interest

The authors have no conflict of interest.

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