Original Research Article

Aged garlic extract potentiates doxorubicin cytotoxicity in human breast cancer cells

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Abstract

Purpose: To investigate the potential chemo-sensitizing effect of aged garlic extract (AGE) on doxorubicin (DOX) in breast cancer cells (MCF-7), and the possible underlying mechanisms.
Methods: Human breast cancer cell line (MCF-7) was treated with AGE and DOX. The cytotoxic effects of AGE and DOX were investigated via cell cycle analysis and apoptosis induction, using flow cytometry. Mechanistic studies involved the determination of cellular uptake of DOX and p-glycoprotein (P-gp) activity.
Results: Combined treatment of MCF7 cells with AGE and DOX produced no significant effect at AGE dose of 10 mg/mL. However, co-treatment with AGE at doses of 50 and 93 mg/mL enhanced the cytotoxicity of DOX on MCF-7 cells, with IC_{50} values of 0.962 and 0.999 µM, respectively, when compared with 1.85 µM DOX alone. Moreover, Annexin V-FITC and PI techniques showed that AGE significantly increased percentage of cells in late apoptosis. Besides, AGE-DOX treatment significantly increased cellular uptake of DOX and inhibited P-gp activity, when compared with DOX alone (p < 0.05).
Conclusion: AGE enhances the cytotoxic effect of DOX on MCF-7 cells, most likely due to cell cycle distribution, stimulation of apoptosis, increased uptake of DOX by MCF7, and inhibition of P-gp activity.

Keywords: Aged garlic extract, Doxorubicin, Breast cancer, MCF-7 cell line, P-glycoprotein, Apoptosis, Cell cycle

INTRODUCTION

Breast cancer (BC) is the most prevalent malignancy in women worldwide. The worldwide incidence of the BC is on the rise [1]. The incidence of BC varies with geographical location, with the highest cases in advanced countries, and lower cases in developing countries in Asia, the Middle East, and Africa [2]. The first line chemotherapeutic drug used for BC is doxorubicin (DOX) which is a broad spectrum anthracycline antibiotic against lymphomas, leukemia, and solid tumors [3]. However, DOX is associated with cardiotoxic side effects which limit its use. In an attempt to develop a new therapeutic combination so as to maximize the

chemotherapeutic effects for DOX at low doses and minimize its adverse effects, a diversity of tactics have been investigated.

It is worth noting that herbal medicines have been used for long in the prevention and treatment of various diseases such as heart disease, cancer and obesity. Garlic (*Allium sativum*) is a member of vegetables belonging to the genus *Allium*. Organosulfur compounds are responsible for the health benefits derivable from garlic consumption. Recently, the therapeutic benefits of garlic in diverse biosystems has been discovered. These benefits include anti-oxidant, anti-tumorigenic and cardio-protective effects [4,5]. Aged garlic extract (AGE) is a liquid prepared through prolonged ethanol extraction of fresh garlic for up to 20 months at room temperature [6]. The extract does not cause adverse events, and it has been confirmed to be safe in preclinical trials [7]. Compared with other garlic products, AGE is the most useful garlic preparation used as an antioxidant [5]. It exerts cytotoxic properties in a wide variety of tumor cells, including gastric and colon cancer cells [8]. In addition, it has been reported that AGE exerted protective effects against DOX-induced cardiotoxicity in rats [4].

Human breast cancer cell lines are fundamental tools for studying BC at the molecular level. They could be utilized as *in vitro* models in laboratory cancer research. The MCF7 cell line was isolated by Dr. Soule and his colleagues in 1973 from the pleural effusion of elderly patient with metastatic breast carcinoma at the Michigan Cancer Foundation. This cell line is accepted globally as an appropriate model for use in the evaluation of the anticancer effects of drugs [9]. The current study was designed to determine the potential chemo-sensitizing effect of AGE on the cytotoxicity of DOX against MCF-7, as well as the possible underlying mechanisms, with respect to cell cycle phase distribution, apoptosis induction, DOX cellular uptake and P-glycoprotein activity.

EXPERIMENTAL

Chemicals

Doxorubicin hydrochloride (DOX), phosphate buffered saline (PBS), culture medium (RPMI 1640), fetal bovine serum (FBS), trypsin/EDTA mixture, penicillin G, streptomycin and rhodaminse 123 were purchased from Merck (St. Louis, MO, USA). Aged garlic extract (Kyolic, AGE) was kindly provided by Wakunaga of America (Mission Viejo, CA).

Cell culture and measurement of cytotoxicity

Human Breast Cancer cell line (MCF-7) was obtained from National Cancer Institute (NCI), Cairo University, Egypt. The cells were cultured in RPMI 1640 supplemented with 10 % FBS, streptomycin (50 μg/ml) and penicillin (100 U/mL) at 37 ˚C in a 5 % CO_2 incubator. Cytotoxicity was determined using SRB assay according to the method of Skehan *et al* [10]**.** The cells were simultaneously incubated with different concentrations of DOX and AGE i.e. DOX at concentrations of 0.1, 1, 10 and 100 μM; and AGE at concentrations of 10, 50, and 93 mg/mL, with 3 wells for each concentration. After 48 h, cell monolayers were fixed with trichloroacetic acid (10 % w/v) and stained with 0.4 % SRB using Cell Cytotoxicity Assay Kits (Aldrich Chem. Corp., USA), in line with the manufacturer's instructions. Optical density was read at 490 nm in a microplate reader (Model ELx808, BioTek, U.S.A.).

Cell cycle analysis

The MCF7 cells were seeded in 6-well plates at a density of 2 x10^5 cells/well in RPMI 1640 for 24 h. Thereafter, the cells were incubated with DOX (1.85 μM) alone and/or simultaneously with AGE (50 and 93 mg/mL). After 48 h, the cells were washed thrice with PBS, followed by harvesting via trypsinization. For cell cycle analysis, the pellet was re-suspended at cell density of 1 x 10^6 cells/mL in the assay buffer, and processed according to the instructions in the cell cycle determination kit (Cayman Chemical Company, USA). Cell cycle analysis was carried out using flow cytometry (Becton Dickinson (BD) FACS Caliber, U.S.A) [11].

Determination of apoptosis

Apoptotic and necrotic cells were distinguished and investigated using flow cytometry based on the assay of Van Engeland *et al* [12], using Annexin V-FITC apoptosis detection kit obtained from Aldrich Chem. Corp., USA. The cells were suspended in 200 μL of Annexin V incubation reagent prepared (for each sample) by mixing PI, binding buffer 10X and Annexin V-FITC in deionized water. The mixture was incubated in the dark at 23°C for 15 min, followed by addition of 400 μL of binding buffer to each sample, and flow cytometric analysis (NAVIOS Beckman Coulter, U.S.A.).

Assay of cellular uptake of DOX

After cell treatment and trypsinization, 1 x 10^6 cells were digested by resuspending them in 2

mL of 1:1 (v: v) mixture of ethanol and 0.3 M HCl for 24 h at 70 °C. Then, 3 mL of PBS was added to each sample. In a clear, black flat-bottom 96-well plate, 100 µL of lysed cells was added to each well. The lysed cells were analyzed using a spectrofluorometer (Synergy HT microplate reader, BioTek, USA) at optimal excitation and emission wavelengths of 485 and 590 nm, respectively [13,14].

Assay of P-gp activity

Following 24-hour cell seeding, 100 µL of working solution of Rhodamine 123 (2.62 µM) was added per well and the wells were kept in a CO_2 incubator at 37 °C in the dark for 30 min. Then, the cells were treated as stated previously. Following trypsinization, the cells were washed once with ice-cold PBS. For P-glycoprotein assay, one million cells were suspended in 1 mL of PBS, with shaking. The lysed cells were analyzed using a spectrofluorometer (Synergy HT microplate reader, BioTek, USA) at wavelength range of 485 - 590 [15–17].

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was done using one-way analysis of variance, and then Tukey's *post-hoc* test. All analyses were carried out with InStat version 3 software package. Graphs were drawn by means of GraphPad Prism (ISI® software, USA) version 5 software. Values of $p < 0.05$ were used as criteria for statistical significance of differences.

RESULTS

Effect of DOX and AGE on cytotoxicity of MCF-7 cells

Figure 1 shows the effect of DOX (with or without simultaneous treatment with different concentrations of AGE) on the growth of MCF-7 cell line. Treatment with DOX alone at a dose of 1 µg/mL resulted in IC_{50} value of 1.85 µM. Interestingly, co-treatment of the cells with AGE (10 mg/mL) decreased the sensitivity to the cells to the cytotoxic effect of DOX, with IC_{50} elevated to 9.46 µM.

In contrast, co-treatment with higher concentrations of AGE (50 and 93 mg/mL) significantly increased the sensitivity of MCF-7 cells to DOX, with IC_{50} values of 0.962 and 0.99 µM, respectively, without any obvious difference in cytotoxicity between the two higher concentrations.
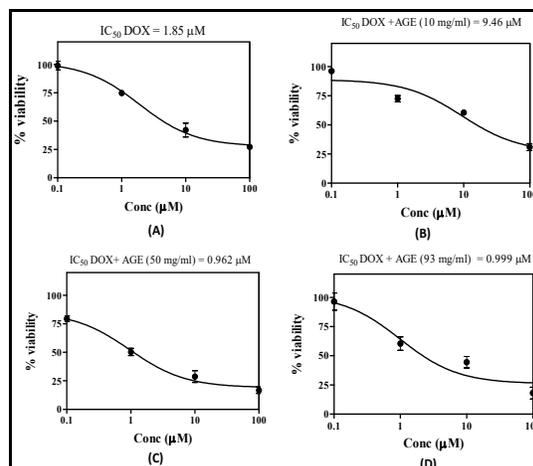

Figure 1: Effect of DOX and/or AGE treatment on the viability of MCF-7 cells. Cell survival was determined after 48 h of exposure to the drugs. Data are presented as mean ± SEM of 3 independent experiments. (A) IC_{50} of DOX alone, (B) IC_{50} of DOX (1.85 µM) + AGE (10 mg/mL); (C) IC_{50} of DOX (1.85 µM) + AGE (50 mg/mL); (D) IC_{50} of DOX (1.85 µM) + AGE (93 mg/mL)

Cell cycle analysis

As shown in Figure 2 and Table 1, treatment with AGE (50 and 93 mg/mL) showed significant increase in apoptosis (9.87 and 15.13 %, respectively), when compared to apoptotic cells in the control (7.03 %). Moreover, the higher AGE dose (93 mg/mL) resulted in a significant decrease in the proportion of cells in the S and G2/M phases (23.53 and 11.87 %, respectively), when compared to cells in the S and G2/M phases in the control group (28.83 and 17.97 %, respectively).

Combination treatment with DOX (1.85 µM) and AGE (50 and 93 mg/mL) produced significant increases in apoptosis (16.37 and 23.37 %, respectively), when compared with control (7.03 %) and DOX alone (12.5 %). Furthermore, combination treatment with DOX (1.85 µM) and AGE (93 mg/mL) resulted in significant decrease in cells arrested in G1/G0 phase of the cell cycle (24.07 %), when compared with control (41.83 %) and DOX alone (38.7 %). Moreover, this combination produced significant decrease in cells in S phase (23.87 %), when compared with DOX alone (30.47 %). On the other hand, it resulted in significant increase in cells accumulated in G2/M phase of the cell cycle (20.73 %), when compared with DOX alone (14.43 %). Interestingly, the combination of DOX (1.85 µM) and AGE (50 mg/mL) significantly increased apoptotic cells by more than two folds, while combination of DOX (1.85 µM) and AGE (93 mg/mL) significantly increased apoptotic cells

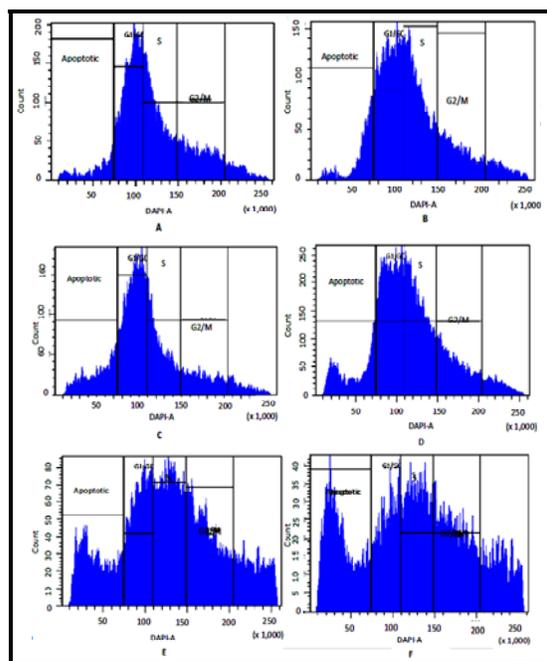by more than three folds, when compared to the corresponding controls.

Figure 2: Effect of DOX and /or AGE treatment on cell cycle phase distribution of parent MCF-7 cells. Cell cycle distribution was analysed after the cells were exposed to the drugs for 48 h and stained with PI. (A) Control, (B) cells treated with DOX (1.85 µM), (C) cells treated with AGE (50 mg/mL), (D) cells treated with DOX (1.85 µM) plus AGE (50 mg/mL); (E) cells treated with AGE (93 mg/mL), and (F) cells treated with DOX (1.85 µM) plus AGE (93 mg/mL).

Effect of treatments on apoptosis

Figure 3 shows the distribution of cells in each quadrant according to necrosis, late apoptosis, live cells, and early apoptosis (Annexin V-positive cells). Cell treatments with DOX and/or AGE were carried out in a way similar to that used in cell cycle analysis. As shown in Figure 3, the most prominent effect was increase in percentage of cells in late apoptosis quadrant.

The percentages of live and late apoptotic cells after treatments are shown in Table 2. The percentage of live cells was significantly decreased by more than 50 % after treatment with DOX (1.85 µM), when compared with control cells. The effect of AGE alone (50 mg/mL) was weaker than that of DOX: although it was able to significantly reduce live cells, it did so at a lower extent than DOX. However, more obvious effects were observed after co-treatment with DOX (1.85 µM) and AGE (50 mg/mL), where live cells decreased to almost one-third of the control value. Amazingly, combination treatment of DOX (1.85 µM) and AGE (93 mg/mL) produced very strong effect.

The percentage increase in late apoptotic cells was dramatic after treatment with 1.85 µM DOX (45 %) or AGE at doses of 50 and 93 mg/mL (19.73 and 48.5 %, respectively), when compared to the control (7.2 %).

These findings confirmed that apoptosis was significantly elevated after treatment with DOX. Again, co-treatment with DOX (1.85 µM) and AGE (50 mg /ml) was more effective in increasing percentage of apoptotic cells than treatment with DOX alone. In addition, cell treatment with AGE alone (93 mg/mL) resulted in late apoptosis in almost half of the cell population. Furthermore, co-treatment with DOX and AGE (93 mg/mL) had very potent effect, being able to induce apoptosis in more than 90 % of the cell population.

Effect of treatments on DOX cellular uptake and p-gp activity

To determine the sensitivity of MCF-7 cells to the growth-inhibitory effect of DOX against MCF-7 cells, intracellular DOX level per 10^6 cells was measured. The cells were treated with DOX (1.85 µM) in the presence or absence of AGE (50 and 93 mg /mL).

Table 1: Effect of AGE and/or DOX treatment on cell cycle phase distribution of MCF-7 cells

Treatment	Apoptotic	G0/G1	S	G2/M
Control	7.03 ± 0.4	41.83 ± 1.15	28.83 ±1.53	17.97 ± 0.4
DOX (1.85 µM)	12.5[a] ± 0.66	38.7 ± 1.73	30.47 ± 1.11	14.43 ± 1.55
AGE (50 mg/mL)	9.87[a] ± 0.67	36.3 ± 4.88	32.63 ± 4.6	15.87 ± 1.16
DOX (1.85 µM) + AGE (50 mg/mL)	16.37[a, b] ± 0.96	32.53[a] ± 3.1	27.5 ± 1.04	17.83 ± 2.68
AGE (93 mg/mL)	15.13[a] ± 0.35	46.8 ± 2.25	23.53[b] ± 0.97	11.87[a] ± 0.45
DOX (1.85 µM) + AGE (93 mg/mL)	23.37[a, b] ± 1.96	24.07 [a, b] ± 1.76	23.87[b] ± 0.75	20.73[b] ± 3.35

Data are presented as mean ± S.E.M of three independent experiments (n = 3). Statistical analyses were performed using one-way ANOVA, followed by Tukey's *post-hoc* test. [a]$p < 0.05$, compared with corresponding control; [b]$p < 0.05$, compared with corresponding DOX-alone treatment
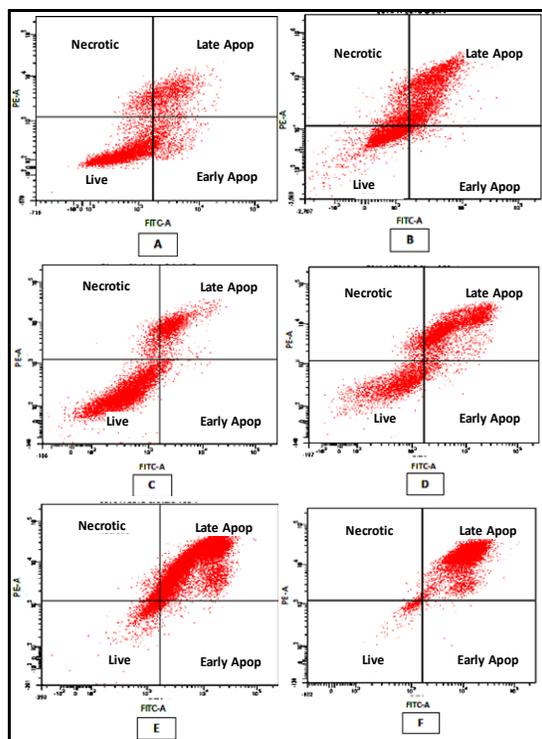
Figure 3: Effect of AGE and /or DOX treatment on induction of apoptosis in MCF-7 cells. Apoptosis was analysed after the cells were exposed to the drugs for 48 h and stained with Annexin V-FITC and PI. (A) control, (B) cells treated with DOX (1.85 µM), (C) cells treated with AGE (50 mg/mL), (D) cells treated with AGE (50 mg/mL) plus DOX (1.85 µM), (E) cells treated with AGE (93 mg/mL), and (F) cells treated with AGE(93 mg/mL) plus DOX (1.85 µM)

Figure 4 A shows that DOX cellular uptake/10^6 cells was increased significantly by more than 2 folds on co-treatment with AGE (50 mg/mL). Moreover, co-treatment with DOX and AGE (93 mg/mL) caused about 4-fold increase in DOX uptake by MCF-7 cells. In order to explain the higher cellular uptake of DOX in the presence of AGE, P-gp activity was assayed via determination of the accumulation of Rh123 in MCF7 cells. As indicated in Figure 4 B, Rh123 accumulation was markedly increased in MCF-7/DOX cells in the presence of AGE, when

compared to MCF-7/DOX cells. This effect was concentration-dependent, since AGE at a concentration of 50 mg/mL, increased intracellular Rh123 by about 3.8 folds, while AGE at a concentration of 100 mg/mL, had a more prominent effect, being able to cause > 6-fold increase of Rh123 accumulation, when compared to the control value. These results suggest that P-gp active outward transport was inhibited by AGE.
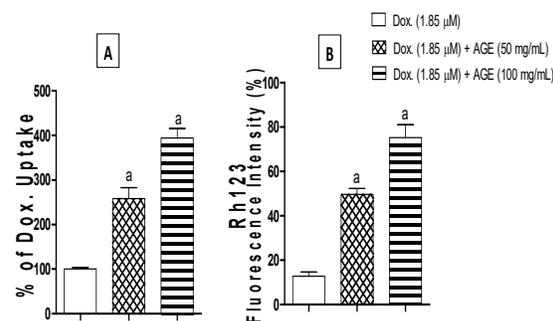

Figure 4: Effect of AGE co-treatment on: (A) cellular uptake of DOX/10^6 MCF-7 cells, (B) P-gp activity, as indicated by intracellular accumulation of Rh123/10^6 MCF-7 cells. Both assays were carried out spectrofluorometrically. Data are displayed as mean ± S.E.M of 3 independent experiments (n=3). Statistical analyses were performed using one-way ANOVA, followed by Tukey's *post-hoc* test; [a]$p < 0.05$, compared to DOX

DISCUSSION

Doxorubicin (DOX) is widely used in cancer therapy for human neoplasms. The clinical benefits of DOX are limited by cell resistance and serious adverse effects, specifically dose-related and cumulative cardiotoxicity [18]. Chemo-sensitization is a strategy for overcoming chemoresistance. A diversity of tactics has been employed to enhance the therapeutic effects of chemotherapeutic agents, while decreasing their toxicities. Among the potential chemosensitizers is the natural product AGE, which exerts chemo-preventive and cytotoxic effects.

Table 2: Effect of DOX and /or AGE treatment on live cells and late apoptosis in MCF-7 cells

Treatment	Live cells	Late apoptosis
Control	83.87 ± 1.45	7.2 ± 0.71
DOX (1.85 µM)	39.17[a] ± 2.53	45 [a] ± 2.7
AGE (50 mg/mL)	71.17[a, b] ± 1.68	19.73 [a, b] ± 1.86
DOX (1.85 µM) + AGE (50 mg/mL)	33.1[a, b] ± 3.3	56 [a, b] ± 2.91
AGE (93 mg/mL)	36.3[a] ± 1.6	48.5 [a] ± 1.57
DOX (1.85 µM) + AGE (93 mg/mL)	3.4[a, b] ± 0.26	91.5 [a, b] ± 1.99

Data are shown as mean ± SEM (n = 3). Statistical analyses were performed using one-way ANOVA, followed by Tukey's *post-hoc* test. [a]$p < 0.05$, compared to control; [b]$p < 0.05$, compared to corresponding DOX-alone treatment

This study was aimed at investigating the potential chemo-sensitizing effect of AGE on DOX against the growth of MCF-7 cells, as well as the likely underlying mechanisms with regard to DOX cytotoxicity, cell cycle phase distribution, apoptosis induction, DOX cellular uptake and P-glycoprotein activity. Treatment of MCF-7 cells with different concentrations of DOX alone was cytotoxic to the cells, with IC$_{50}$ of 1.85 μM (equivalent to 1 μg/mL). Similar findings were reported by Buranrat and co-workers who exposed MCF-7 cells to DOX and determined their viability using SRB assay [19]. Their results demonstrated that cell growth was inhibited after 48 h of treatment, with IC$_{50}$ value of 1.8 ± 0.1 μM at 48 h.

In the present study, DOX cytotoxicity was established in the results of cell cycle distribution and induction of apoptosis, where DOX (1.85 μM) caused a significant increase in apoptotic cells, significant decrease in the percentage of live MCF-7 cells, and marked increase in the extent of apoptosis, when compared with control cells. Barzegar *et al* reported that DOX efficiently arrested cell division and stimulated apoptosis via DNA intercalation, generation of oxidizing moieties, binding to cellular membranes, and inhibition of topoisomerase II (Topo IIA) which is greatly expressed in S and G2/M phases [20].

Approaches based on making good use of natural products for cancer prevention and/or treatment have attracted a lot of attention in the past years. Garlic (*Allium sativum*) has been known for long for its medical uses. Multiple reports suggest that AGE exerts anti-cancer effect with multiple cellular mechanisms such as induction of apoptosis, cell cycle arrest, and suppression of cell proliferation [11]. Inhibition of early and late stages of cancer by AGE leads to inhibition of tumour growth in many tissues like mammary gland, skin, colon, and gastric tissue [21].

The anticarcinogenic effect of AGE is attributed to its richness in organo-sulfur compounds such as allicin, S-allyl-cysteine, S-allo-mercapto-cysteine, diallyl-trisulfide and diallyl-disulfide. Thus, AGE could be considered as a perfect sensitizer to the cytotoxic effect of DOX against breast cancer cells. In the present study, higher concentrations of AGE were more cytotoxic than the lower concentration. As reported by Pourzand *et al,* high intake of garlic is linked to decreased risk of breast cancer [21]. In the current study, MCF-7 cells treated with AGE alone at higher concentrations (50 and 93 mg/mL) showed a significant increase in apoptotic cells. Aged garlic extract (AGE) has

been previously reported to induce dose-related cell cycle arrest, growth inhibition and apoptosis in many human cancer cell lines [22].

To understand the mechanism of interaction between DOX and AGE, apoptosis assay, cell cycle assay and DOX cellular uptake assay were performed. Apoptosis assay showed that DOX-AGE combination significantly increased apoptotic cells, and significantly decreased the population of cells in G1/G0 phase, when compared with DOX alone. These results are in agreement with those of Zhang *et al*, who reported that SAMC derived from garlic effectively inhibited the growth of MCF-7 cells via stimulation of apoptosis and cell cycle arrest at G0/G1 phase. They also suggested that SAMC-induced apoptosis in MCF-7 (estrogen-dependent) and human hormone-independent breast cancer (MDA-MB-231) cells occurred through activation of the mitochondrial apoptotic pathway via upregulation of Bax, downregulation of Bcl-2 , and activation of caspase-9 and caspase-3 [22].

In this study, the percentage of cell accumulation at S phase was significantly decreased in combination treatment with DOX and highest concentration of AGE, when compared to the DOX alone. This finding is in agreement with that of Osman *et al*, who reported that resveratrol induced cell cycle arrest at S phase, thereby exposing a high percentage of tumour cell population to DOX so that more cells underwent apoptosis and entered G0 phase [23]. The results were further strengthened by the rise in DOX cellular uptake following co-treatment with AGE in a concentration-related manner. There was progressive build-up of DOX in cells co-treated with DOX/AGE, which is consistent with exposing higher proportion of MCF-7 cells to DOX through cell accumulation in G0 phase. The rise in DOX MCF-7 uptake might be based on the inhibition of P-gp and multidrug resistance which are involved in DOX absorption, distribution and elimination [24].

In the current study, Rh123 efflux was suppressed in DOX-treated MCF-7 cells in the presence of AGE. This finding is in agreement with that of Abdallah *et al*, who reported that resveratrol inhibited P-gp, upon co-treatment with DOX, thereby triggering a rise in cellular uptake of DOX [24]. It has been reported that AGE suppressed the energy-dependent efflux pump of P-gp, thereby producing an augmented intracellular drug concentration and increasing cellular toxicity [25]. The extract prevented further absorption of DOX in cardiac tissue (cardio-

protection), but it increased DOX concentration in tumour cells.

CONCLUSION

Aged garlic extract (AGE) has a chemo-sensitizing effect on DOX in human breast cancer cell line (MCF-7). This effect could be attributed to induction of apoptosis and enhanced intracellular DOX accumulation, the latter of which results from inhibition of P-gp activity.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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