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Original Article

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Abstract

Over the last decades, cancer stem cells (CSCs) have been a major hurdle challenging every oncologist worldwide. Despite the anticancer effects of the adjuvant therapy protocols, they still facing major challenges include harmful side effects and poor free survival period that is ascribed to the EMT and metastasis. Recently, alternative approaches of treatment using plant extracted phytochemicals that originally exist in the dietary food and plant roots have proved their anticancer effects with extra advantage that they have no side effects on the healthy organs. In the current study, a combination of phytochemicals has been evaluated as an anticancer, anti-metastatic drug for the highly invasive breast cancer cells MDA-MB231. The data revealed that the low dose of the combination has significantly decreased the expression of the migratory genes (N- Cadherin and Vimentin) after 72hr of exposure to the combination. Furthermore, the low dose has also reduced the proliferation rate of the MDA-MB231 cells after 6 days. This study demonstrates that using low doses of phytochemicals for a longer time may be a promising protocol for inhibiting cancer proliferation and metastasis.

1. Introduction

Breast cancer has the highest rate of mortality in women diagnosed with cancers worldwide. It accounts for 25% of all types of cancers **(Fidler *et al.*, 2017).** According to the National Cancer Institute-Cairo University (NCI-CU), 19.3% of all the primary malignancies in Egypt are breast cancer cases **(Naglaa et al., 2018).** Breast cancer is a heterogeneous tumor on the molecular, pathological and epidemiological level that is divided into many subtypes. The current protocols for breast cancer varies between traditional adjuvant and neo-adjuvant therapies (surgery, radiotherapy, Chemotherapy and endocrine therapy) **(Maughan *et al.*, 2010).** Despite cytotoxic drug's efficiency in eradicating primary tumors, we still have no control on the metastatic tumors that initiate secondary tumors in distant organs **(Li and Kang, 2016).** Besides most of the traditional treatments have many limitations including major side effects in different healthy organs, as they unselectively target all proliferative and growing cells including both normal and tumoric cells as long as the continuous uptake of these drugs leads to drug resistance**(Diaby *et al.*, 2015)**. Therefore, it is a critical challenge to develop new and alternative approach of treatment that is more selective to the cancer cells to reduce the side effects on the patients. Recent studies have investigated the role of natural extracts from plants and the dietary food in inhibiting cancer progression pre-clinically and clinically **(Zubair *et al.*, 2017; Choudhari *et al.*, 2020).** Our team has reached a combination of phytochemicals exists naturally in food such as vegetables, fruits, spices and plant roots which is "curcumin, indol-3-carbinol, resveratrol, C-phycocyanin, quercetin, gallic acid, genistein". Firstly we have investigated the role of six combined phytochemicals in the multiple targeting of several signaling pathways at the same time to inhibit the cancer proliferation in vitro. The data revealed that there is a significant reduction in BCL2, SVV, CD44, CDK4 and the mutant P53 in the transcriptional level, indicating the efficacy of this combination on inhibiting cancer proliferation, inducing apoptosis and limiting cancer invasion in both primary and metastatic breast cancer cell lines. Interestingly, this combination has been tested on normal mesenchymal stem cells as indicator for its selective cytotoxicity for the cancer cells only

(Ouhtit *et al.*, 2013). Recently, the combination has been investigated as an adjuvant treatment with the Ionizing Radiations (IR) on liver cancer in vitro, and we found that the combination has the potential to sensitize the cancer cells to the IR treatment through its potent antioxidant activity which lead to the diminish of liver cancer cells **(Abdraboh *et al.*, 2020).** In this study, we aim to investigate the possible synergetic effect of the combination on the proliferation and migration of the metastatic breast cancer cell line MDA-MB231.

2. Materials and Methods

2.1. Dose- time dependent MTT:

MDA-MB 231 cells were seeded in 96-well plate as ($1*10^3$ cells/well) in 200µl of complete medium. After 24hr of incubation, the following doses of the combination were added to the cells in triplicates (1080µg/ml and 54µg/ml) and incubated for 2, 4 and 6 days at 5% CO2 and 37ºC. The reduction in cell proliferation was calculated according to manufactures' protocol (MTT, Serva, Electrophores GmbH, Germany). The percentage of viable cells was correlated to the level of dye reduction measured at 570nm (BioTek, Gen5™, 11660241).

2.2. Colony forming assay:

MDA-MB231 cells were seeded in 6-well plate as ($1*10^3$ cells/well) in 2ml of complete medium. After 24hr the following doses of the combination were added (1080µg/ml and 54µg/ml). After incubation for 11 days until the survived cells developed colonies countable by naked eye. Cells were fixed with methanol then stained with crystal violet. Colonies of at least 50 cells were counted.

2.3. Wound healing assay:

MDA-MB231 cells of control, 1080µg/ml, 54µg/ml groups were cultured in 6-well plate as ($2*10^5$ cell/well) and left until they became 100% confluence. A scratch with 100µL micro-pipette tip was induced diagonally in the middle of each well. Then, cells were washed twice with PBS to remove the detached cells, and they were incubated for 16hr. Microscopic photographs were taken using inverted microscope at 0hr and 16hr.

2.4. Dose- time dependent PCR:

Total RNA was extracted from MDA-MB231 cells using the RNeasy Mini Kit (Qiagen, Germany, cat. no.74104) according to manufacturer's protocol. The quality and concentration of the extracted RNA was detected by NanoDrop ND-2000 spectrophotometer (Thermo Fisher, DE, USA). Complementary DNA was synthesized from 1 µg of total RNA using (RevertAid First Strand cDNA Synthesis, Thermo Scientific, USA, K1622) according to the manufacturer's protocol. Determination of the expression levels were assessed using Thermo Scientific DreamTaq Green PCR Master Mix (2X) according to manufacturer's protocol (Thermo Scientific, CA, USA) using the following primers: Vimentin (sense) '5-TGT CCA AAT CGA TGT GGA TGT TTC-3′, (antisense) 5′-TTG TAC CAT TCT TCT GCC TCC TG -3′, N-Cadherin: (sense)5′- GAG ATC CTA CTG GAC GGT TCG -3′, (anti- sense) 5′- TCT TGG CGA ATG ATC TTA GGA -3′, E- Cadherin: (sense)5′- GAG TGC CAA CTG GAC CAT TCA GTA -3′, (antisense) 5′- AGT CAC CCA CCT CTA AGG CCA TC -3′ and GAPDH: (sense)5′- ACC ACA GTC CAT GCC ATC -3 (antisense) 5′- TCC ACC ACC CTG TTG CTG TA-3′. Thermal cycling conditions were conducted by initial heating step at 95ºC for 10 min. followed by 35 cycles at 95ºC for 30 s, annealing at 60ºC, 55ºC, 60.4ºC and 60ºC for Vimentin, N- Cadherin, E- Cadherin and GAPDH, respectively, for 30s each and 72ºC for 1min and final extension at 72ºC for 10s. Relative expression of Vimentin, N- Cadherin and E- Cadherin mRNA was normalized against GAPDH transcript as an internal reference control.

2.5. Statistical analysis

Data were expressed as mean ± SEM, Student's t-test was used to compare the mean differences between samples using Graphpad Prism software version 6.01 (GraphPad Software, CA, USA). For all analyses, $P < 0.05$ was considered statistically significant.

3. Results

3.1. The combination inhibited cell growth time dependently:

Time dependent inhibition percentage of the combination is associated by the reduction in cell proliferation of MDA-MB231 as it's showed in **Figure (1).** The efficacy of the doses increases by time as the most effective time point was after 6 days as the inhibition reaches approximately (93% and 61%) for (1080µg/ml and 54µg/ml) respectively.

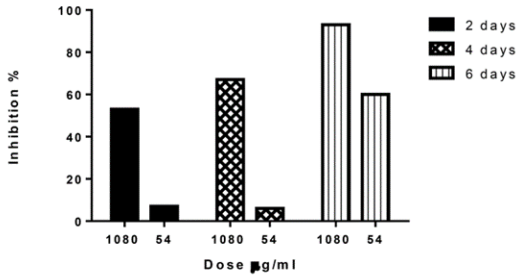

Figure (1): Time-dependent inhibition of MDA-MB231 cell growth of cells. MTT assay indicated a significant time dependent inhibitory effect of the phytochemical's combination on the growth of MDA-MB231 cell line. The presented data are the means of three separate experiments.

3.2. Effect of the combination on MDA-MB231 cell migration:

The combination has shown a potent effect in inhibiting the highly metastatic MDA-MB231 cell line to migrate. That was shown in the inability of the treated cells to close the wound compared to the untreated control that closed the wound at 16hr, as it is shown in Figure (2A, 2B).

3.3. Gene expression of EMT markers:

Gene expression profile for the EMT genes (N-Cadherin and Vimentin) revealed the expression of both genes has decreased in the group treated with

(54µg/ml) specially after 72hr of exposure to the combination compared to the untreated control group, **Figure (3A, 3B).**

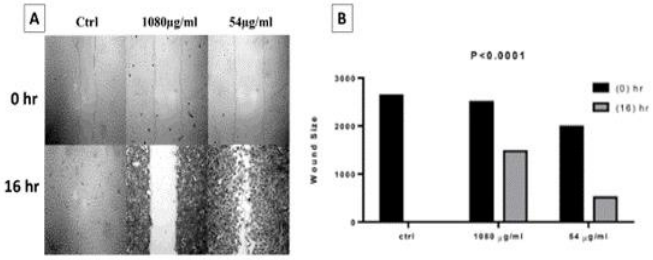

Figure 2: (A) The inhibitory effect of the combination on cell motility of the highly metastatic MDA-MB231 cell line, after incubation for 16 hr at different concentrations (1080µg/ml, 54µg/ml and Ctrl). The wound size was calculated using Image J software. (B) histogram showing the significant effect of the combination on diminishing the migration of MDA-MB-231 cell with significance ***P< 0.0001.

3.4. The combination inhibited MDA-MB231 cells from forming colonies:

The results revealed that the combination has inhibited the MDA-MB231 cells from forming colonies in the group treated with (54µg/ml) compared to the untreated control cells as it's shown in **Figure (4A, 4B).**

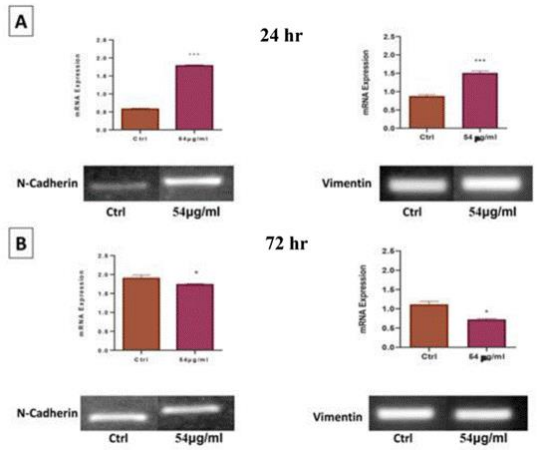

Figure (3): (A) The dose-time dependent PCR analysis of the metastatic genes (N Cadherin and Vimentin) at 24hr showed significant increase in the expression of both genes. (B) The dose-time dependent PCR analysis of the metastatic genes (N Cadherin and Vimentin) at 72hr showed attenuated expression of both genes, the thickness of the PCR bands was measured using Image J software.

4. Discussion

Over the last decades, cancer has been a major hurdle challenging every oncologist worldwide. Furthermore, breast cancer is the most common type of cancers that has become the leading cause of mortality and morbidity among women **(Fidler *et al.*, 2017).** Triple Negative Breast Cancer (TNBC) is one of the most aggressive and invasive types of breast cancer; it's known by its ability to migrate into distant organs forming secondary tumors through the EMT process**(Carey *et al.*, 2010).** Traditional treatments of breast cancer have proven their efficiency in diminishing the cancer population but, still metastasis is the most challenge facing them **(Li and Kang, 2016).**

The Greek philosopher Hippocrates which considered as the god father of medicine; has stated two and half thousand years ago "let food be the medicine and medicine be the food" which is recently being elucidated by the 50% reduction at cancer incidence among persons that daily consumes at least five serving of vegetables and fruits **(Dave et al., 2020).**

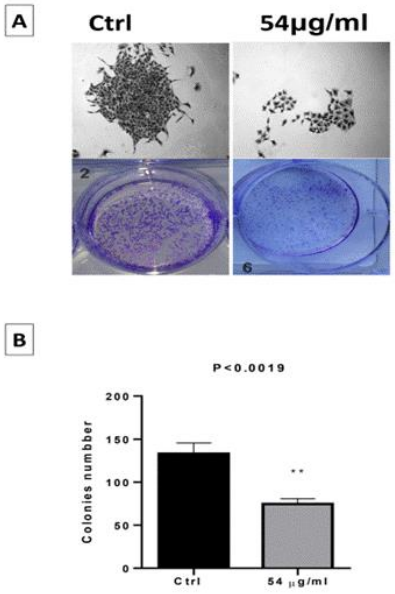

Figure 4: (A) Effect of the combination on the ability of MDA-MB231 cells to form colonies. (B) Statistical analysis of the colony formation assay was calculated using GraphPad Prism 8, the significance was denoted as

Currently, several preclinical studies have illustrated the significant effect of nutritional and dietary factors on the incidence and prevalence of some cancers pointing out the increased interest in dietary phytochemicals implication in clinical trials. Even though, one of the main tackles in implication of phytochemicals in clinical trials is their need to be served in high doses that are not achievable in such large portions of food **(Rizeq et al., 2020).** Additionally, several studies have discussed the role of the dietary phytochemicals int the modulation of various signaling transduction pathways **(Zhao et al., 2018).**

In this study we aimed to investigate the potentiality of a combination of phytochemicals on the proliferation and metastatic ability of the highly invasive MDA-MB231 breast cancer cell line using a low dose of the combination to avoid the cytotoxicity on the normal healthy cells. Recently, the combination has showed its potential antioxidant activity that worked synergistically with IR in

eradicating the liver cancer population **(Abdraboh *et al.*, 2020).**

In order to test the effect of the combination on inhibiting cell proliferation, dose and time dependent MTT assay was applied. The results revealed that the combination has inhibited the proliferation of MDA-MB231 cells. Astonishingly, the effect of the low dose of the combination (54µg/ml) has increased to reach 61% of inhibition after 6 days of exposure (fig. 1). Additionally, the low dose of the combination significantly affected the colony formation ability of the cells compared to the healthy untreated control (fig. 4). Intriguingly, both doses (1080µg/ml and 54µg/ml) have inhibited the cells to migrate and close the wound compared to the control cells that were able to close the wound after 16hr (fig. 2). These data were confirmed by dose- time dependent PCR in which, the low dose of the combination (54µg/ml) has significantly decreased the expression level of EMT genes N-cadherin and Vimentin after 72hr of exposure to the combination (fig. 3).

In summary, the present study demonstrated the efficacy of a low dose of a combination of phytochemicals as a potent anticancer drug that not only inhibited MDA-MB231 cell proliferation but also affected the migration ability of the cells. Thus, this combination could be proposed as a promising therapeutic strategy for triple negative breast cancer, which should be considered for application in upcoming clinical trials.

**P<0.002.

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