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The potential activity of curcumin and resveratrol with abemaciclib in breast cancer cell line

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Abstract
Background and aim: *breast cancer is one of the highly predominant types of invasive cancer and considered one of the main causes of mortality among women. Using combination of drugs in a treatment protocol enhances the therapeutic value of the chemotherapeutic agent. Here, we intended to investigate whether, and how, curcumin (CUR) and resveratrol (RES) give potential therapeutic value to known chemotherapeutic agent abemaciclib (ABEM).**Methods:** using breast cancer cell line MCF-7, we added CUR and RES to ABEM (62.75 μM) for 72 h. The cell growth inhibition of the drugs combination was estimated using cell viability test. Gene expression of miR-4270, vascular endothelial growth factor (VEGF) and p53 as well as levels of nuclear factor-kappa B1 (NF-κB1) and caspase-3 activity were measured. Also, levels of MDA, NO, GSH and SOD activity were estimated. **Results:** combination of CUR and RES synergized the cell growth inhibition effect of ABEM with combination index 0.86. Addition of CUR and RES to ABEM significantly reduced the gene expression levels of miR-4270, VEGF. Also, levels of (NF-κB1), malondialdehyde (MDA) and nitric oxide (NO) were deceased in combination group compared to ABEM treated alone. Whereas, the levels of p53, caspase-3 activity, glutathione (GSH), and superoxide dismutase activity (SOD) were increased in combination group compared to ABEM treated alone. **Conclusion:** treatment of human breast cancer cell line MCF-7 with the combination of CUR and RES could synergize the cell growth inhibition effect of the chemotherapeutic agent (ABEM) alone which may be via their apoptotic, antioxidant, and antiproliferative properties.*
Keywords*: Curcumin, Resveratrol, Abemaciclib, miR-4270, Apoptosis.*

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Abbreviations:
ABEM: Abemaciclib.
CDK4: cyclin-dependent kinase.
CUR: Curcumin.
RES: Resveratrol.
VEGF: vascular endothelial growth factor.
NF-κB1: Nuclear factor-kappa B1.
MDA: Malondialdehyde.
NO: Nitric oxide.
GSH: reduced glutathione.
SOD: superoxide dismutase.

Highlights
- Curcumin and Resveratrol are natural product, have many medicinal activity.
- The combination of Abema with CUR and Res produces a synergistic effect in the breast cancer cell.
- Curcumin and Resveratrol have a prophylactic effect against cancer.
- Gene expression of miR-4270, vascular endothelial growth factor (VEGF) and p53 as well as levels of nuclear factor-kappa B1 (NF-κB1) and caspase-3 activity were measured.

I. Introduction

Breast cancer considers the second main cause of death among females, and is a diverse disease with a high spread rate among worldwide.(Hamam et al., 2016) In order to increase the chance of patient recovery, it is important to understand the biology of breast cancer and to determine new biomarkers for early diagnosis.(Dubey et al., 2015)

Traditional chemotherapy is a remarkable therapy for many tumors in spite of that many patients experience unacceptable toxicity.(Xu et al., 2016) ABEM is a CDK4/6 inhibitor used as a therapy for breast cancer.It was shown previously that ABEM combined with a hormonal therapy enhances its anti-tumor effects.(O'Brien et al., 2018)

Many studies have reported that natural products have a wide spectrum of bioactivities, like the promotion of the immune system, antioxidants and anti-cancer effects.(Miyata, 2007) Moreover, chemoprevention, a new approach to reduce cancer incidence, includes the use of natural products to invert, inhibit or suppress premalignancy before the development of tumor cells.(Rajamanickam and Agarwal, 2008)

Several natural products including curcumin (CUR) have widespread biological use, including the prevention of cancer. CUR exerts its activity by several pathways and may stimulate apoptosis in cells of breast cancer by adjusting the expression of the genes and proteins responsible for apoptosis.(Wang et al., 2016) Recent studies reported that CUR can promote apoptosis in cells of breast cancer by increasing the expression levels of p53, which induces Bax, leading to an increased Bax/Bcl-2 ratio. This cascade results in programmed death of cells of breast cancer.(Choudhuri et al., 2002)

Resveratrol (3,4,5-trihydroxy-trans-stilbene) is a naturally active polyphenol that is produced from the Japanese knotweed. It has been shown previously that it possesses anti-inflammatory, cardio-protective and anti-cancer properties. It is a favorable natural product in the prevention and treatment of chronic inflammatory diseases.(Cui et al., 2010)

MicroRNAs (miRs) provide an unparalleled and worthy biomarker for diagnosis and/or prognosis for some types of cancers.(Wang et al., 2018)miRNAs have regulatory functions in major important processes like cell differentiation, proliferation, and apoptosis. Thus, any alteration in the miRNAs expression mayaffect cellular behavior.(Fan et al., 2013) There are many circulating miRNAs, such as miR-21, 195, 200b, 145, 155 and 200c, that have been determined to be potential breast cancer biomarkers.(Tuna et al., 2016) It has also been shown previously that the expression of miR-4270 is increased in the blood of breast cancer patients.(Hamam et al., 2016) Expression levels of microRNA-4270 has also been reported in patients with breast invasive ductal carcinoma.(Babaei et al., 2018)

Vascular endothelial growth factor (VEGF) regulates the existence and development of cancers via its different receptors, however, the main molecular mechanisms are still unknown.(Luo et al., 2016)Upregulation of VEGF was reported in mouse and human mammary cancer cells.(Gonzalez-Perez et al., 2010) The main biological function of VEGF in breast cancer may not be restricted to angiogenesis. VEGF-signaling in breast cancer is required for the breast cancer cells to avoid apoptotic mechanisms and to be able to proceed towards the metastasis processes.(Mercurio et al., 2005) Apoptosis is a mechanism that plays critical roles in the growth of embryonic cells and tissue homeostasis. Its dysregulation may cause tumor incidence,(Johnstone et al., 2002) therefore, apoptosis evasion is a desirable pathway to discover and design cancer treatment procedure.(Liu et al., 2011)

In the current study, we investigated the additive effect of CUR and RES with ABEM against breast cancer cells through different pathways, such as apoptotic, antioxidant, and antiproliferative mechanisms.

II. Materials and Methods

Chemicals

Abemaciclib (Verzena®) was purchased from Eli-Lilly Company (Ireland). CUR, fetal bovine serum (FBS) and RES were purchased from Sigma-Aldrich (St. Louis, USA). Eagle's Minimum Essential Medium (EMEM), penicillin, and streptomycin were purchased from Biovision Technology Supplies (Cairo, Egypt). All chemicals were of analytical grade.

Cell culture

Breast cancer cell lines MCF-7 and normal MRC5 cells were procured from the American Type Culture Collection (USA). The cells were cultured in 100 cm^2 flasks in EMEM grown with 10 u/ml penicillin, 1.0 µ/ml streptomycin, 2 mM glutamine, 0.1 mM Non-Essential Amino Acids, and 10 % FBS. The cells were incubated in humid weather with 95 % air and 5 % CO_2 at 37 °C. Cell passage was completed according to their rate of division. The medium was changed every 4 days for better growth of cells using 0.25 % trypsin or trypsin/EDTA. The cells passaged after reaching 90 % confluence.

MTT assay

The 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide dye or MTT procedure was used to examine the effects that the extracts had on cellular bioavailability. MCF-7 cells were seeded at 7000 cells/well in a plate with 96-well plates containing 100 µl of EMEM and incubated for 24 h.(Patnaik et al., 2016) Different concentrations were prepared from ABEM (50-100 µmol/L), CUR (5-50 µmol/l) and RES (30-75 µmol/l). MCF-7 cell lines were treated with these different concentrations at 48 h. Cells were protected from light at all times. The medium in the 96-well plates was then carefully removed. We next added 200 µl of MTT (0.5 mg/ml in EMEM) to cells in the 96-well plates and stored them at 37 ºC for 4 h. MTT color was removed and 100 µl dimethyl sulfoxide (DMSO) was added for solubility of the formazan crystals. After incubation at room temperature for 30 m, sample absorption was measured at a wavelength of 570 nm using ELISA reader (Bio Tek Instruments, Inc, USA). The assay was done in triplicates. The percentage of cell viability in the control groups was estimated. The ratio of half median concentration (IC_{50}) was calculated using the Compusyn software Inc.(Rafieian-Kopaei et al., 2014)

Experiment design

Cells were cultured in 100 cm^2 flasks to adhere for 24 h under normal conditions. Afterwards, cells were categorized into six groups. Group I included normal MRC5 cells serving as a negative control. Group II included MCF-7 cells serving as a positive control. Group III included MCF-7 cells that had been incubated with ABEM (62.75 µM) for 72 h. Group IV included MCF-7 cells that had been incubated with ABEM and CUR (17.25 µM) for 72 h. Group V included MCF-7 cells that had been incubated with ABEM and RES (42.5 µM) for 72 h. Group VI included MCF-7 cells that had been incubated with ABEM, CUR and RES for 72 h. The cells were harvested, washed three times in cold PBS, suspended in 500 µl PBS, and kept frozen until biomarker measurement could be completed.(Morisaki et al., 2013) The combination index (CI) was determined using Compusyn software (version 1.0.1).(Chou and Martin, 2007)

RNA Preparation and RT-PCR:

A total RNA Kit (Omega Bio-tek) was used for the separationof total RNA and a Bioron RT/PCR master mix kit (Cat No: 122020-1105) was used to run PCR experiments. PCR was carried outusing Tag master (Jena Bioscience) that produces a high yield of RNA. The cDNA strand was synthesized according to the manufacturer's instructions. The PCR was performed through pre-denaturing for 5 min at 94 °C, then denaturing at 94 °C for 30 sec, followed by annealing at 55 °C and extension at 72 °C for one minute. The amplification was carried out in 30 cycles using BoEco thermal cycler, BYQ6041R-158 (Hamburg, Germany).
NCBI reference sequences were used for preparation of the DNA primers of miR-4270, VEGF, p53 and β-actin respectively.

Table 1: Primers sequences used for the RT-PCR technique.

Primer	Tm (°C)	Sequence (5'----3')
miR-4270		
Forward	57.79	ACAATAGCTTCAGGGAGTCG
Revers	58.05	GACCCACTTTCTTCCCAGC
VEGF		
Forward	59.18	ACCACACCATCACCATCGAC
Revers	59.02	CCCTCCCAACTCAAGTCCAC
p53		
Forward	60.10	CCATCCTCGGTTTCCCTGTC
Revers	60.04	ACAGCAGGAGCAGATTCCAC
β-actin		
Forward	60	CAT GGA TGA CGA TAT CGC TG
Revers	60	CAT AGA TGG GCA CAG TGT GG

Estimation of p53 protein level

A sandwich type of ELISA was used for the quantitative analysis of p53. Briefly, 96-well ELISA trays were coated overnight at 4 °C with 50 µl/well of monoclonal antibody 1801 in PBS (Dynatech Laboratories, UK). Unbound antibodies were washed with the PBS buffer. The wells were incubated for 1 h at room temperature with 200 µl of 5 % BSA in order to block non-specific sites. Wells were then washed with PBS, and incubated for 3 h at room temperature with 50 µl of standard recombinant p53 protein with concentrations of 2.5 ng/ml, 2 ng/ml, 1 ng/ml and 0.5 ng/ml. The wells were incubated for 2 h with 50 µlbiotinylated D07. Wells then were washed and 50 µl of sheep anti-biotin polyclonal antiserum was added and incubated for 1 h with 50 µl of horseradish peroxidase (Stratech Scientific Ltd, Luton, UK). Finally, wells were washed and 50 µl was added of tetra-methylbenzidine substrate. The reaction was stopped with 0.2 M of sulphuric acid. The absorbance was recorded at 250 nm using (Bio Teck Instruments, Inc. plate reader 1603029, USA).The assay was done in triplicates.

Estimation of NF-κB1 protein level

Ray Biotech Cod No: MBS660314, (Georgia, USA) kit was used to determine the NF-κB1 expression level. The lysate cell's supernatants (100 µl) were used for the determination of NF-κB1. The monoclonal antibody (100 µl) was employed for NF-κB1 coated onto the wells provided. The antigen binds to the monoclonal antibody, and then the antibody excess was washed. The ELISA plate was washed three times. An antigen-specific antibody acted as a detector by binding to the antigen marker. A horseradish peroxidase (HRP) antirabbitIgG was used, and then a substrate solution was added to produce color after being acted upon by the peroxidase enzyme. Finally, OD was recorded at (450 nm)within 10 m (Morisaki et al., 2013). ELISA kits were used following the manufacturer's instructions for the detection of the following biomarkers in breast cells. The assay was done in triplicates.

Assay of caspase-3 activity

The cells were lysed with 10 µl of lysis buffer for 30 min at 4 °C and the supernatants were used to determine levels of caspase-3 activity. A colorimetric kit (Code No: CASP-3-C122) was used for the estimation (Sigma-Aldrich, St. Louis, USA), according to the manufacturer's instructions. The absorbance was read at 405 nm using a microtiter plate reader (Bio Tek Instruments, Inc. Highland Park, Winooski, VT, USA). The assay was done in triplicates.

Estimation of oxidative stress markers and antioxidants:

The supernatants of cell lysates were collected. Nitric oxide (NO) and MDA levels are oxidative stress markers. NO levels were estimated using the Griess reaction.(Montgomery and Dymock, 1961) MDA levels in different groups were determined using thiobarbituric acid derivatives.(Uchiyama and Mihara, 1978) The activity of antioxidants, such as GSH and SOD, was estimated. GSH content in cell lysates of each group was estimated using Ellman's reagent.(Ellman, 1959)SOD was estimated using a method which depended on the inhibition of pyrogallol autoxidation.(Marklund and Marklund, 1974) These procedures were estimated using commercially available kits per the manufacturer's instructions (Biodiagnostic, Egypt). Total protein values were found using the procedure described by (Capecchi et al., 1974). The assay was done in triplicates.

Statistical analysis:

Statistical analysis of variance was performed using GraphPad Prism software (GraphPad Inc., version 8.0, CA, USA). Data are presented as the mean ± standard error of mean (SEM), and the levels of significance were accepted at $p<0.05$. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the Tukey's test as a multiple comparison post-ANOVA test.

III. Results

Determination of IC_{50} of ABEM, CUR and RES:

In order to determine the effect of ABEM, CUR and RES on the proliferation of the breast cancer cell line MCF-7, cell growth inhibition assays were conducted using MTT assays. Compusyn software was then employed in order to calculate the IC_{50} of ABEM, CUR and RES. The IC_{50} values for ABEM, CUR and RES were found to be as follows: 62.75, 17.25 and 42.5 µM, respectively (Fig 1). The CI value was calculated by Compusyn software according to the method described by Chou,(Chou and Martin, 2007) in which CI<1 reflects a synergistic effect, CI=1 an additive effect, and CI > 1 an antagonistic effect. Our results showed that the combination of CUR and RES synergists the anti-tumor effect of ABEM with CI value of 0.86.

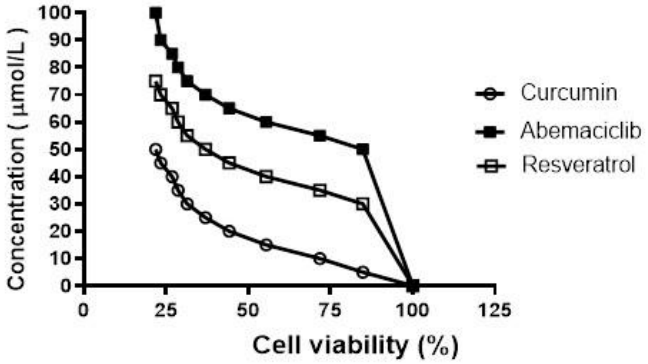

Figure 1: The IC_{50} of ABEM, CUR and RES. Cell viability was assessed by MTT assay. Data points represent the means ± SEM Statistical analysis was determined by one-way analysis of variance (ANOVA), followed by Tukey's as a multiple comparison post-ANOVA test.

Expression of miR-4270, VEGF and p53 by RT-PCR technique

MiR-4270, VEGF and p53mRNA expression levels were evaluated using the RT-PCR technique in all groups of experiment. The expression levels of miR-4270 and VEGF were markedly increased in the positive control group compared to normal cells. The expression of miR-4270 and VEGF was reduced in cells incubated with ABEM compared to the positive control. The addition of CUR and RES to ABEM resulted in a significant reduction in miR-4270 and VEGF expression with respect to cells incubated with ABEM. Incubation of MCF-7 cells with ABEM, CUR and RES resulted in a significant reduction of miR-4270 and VEGF more than other groups (Fig 2 & 3).On the other hand,the p53 expression level was found to be markedly reduced in the positive control as compared to the negative control, whereas its expression was increased in the ABEM group as compared to the positive control. Incubation of MCF-7 cells with a combination of ABEM, CUR and RES resulted in the significant reduction of p53 expression more than in the other groups (Fig 4). The result ofp53 was supported by measuring p53 in the level of protein, which gave a similar result (Table 1). Finally,the expression of β-actin was used as a housekeeping gene in all experimental groups. The expression of β-actin was constant in all groups, which reflects the equal loading of the different samples in all experimental groups.

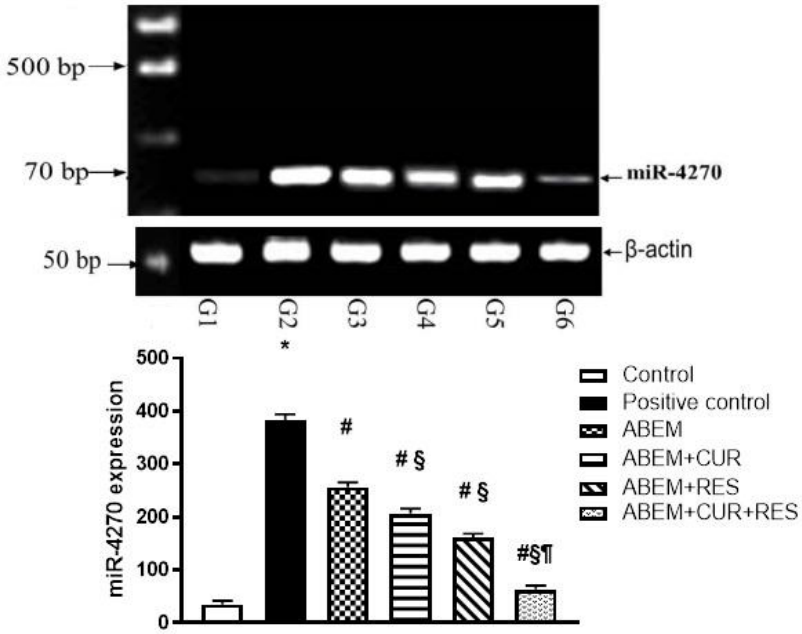

Figure 2: Effect of ABEM and its combination with CUR and/or RES on the expression of miR-4270 in the harvested cells. Data were expressed as mean±SEM. * Values differ significantly from negative control ($p < 0.05$).# Values differ significantly from positive control ($p < 0.05$).§ Values differ significantly from ABMA ($p < 0.05$).¶ Values differ significantly from ABMA & RES ($p < 0.05$).Statistical analysis was determined by one-way analysis of variance (ANOVA), followed by Tukey's test as a multiple comparison post-ANOVA test.

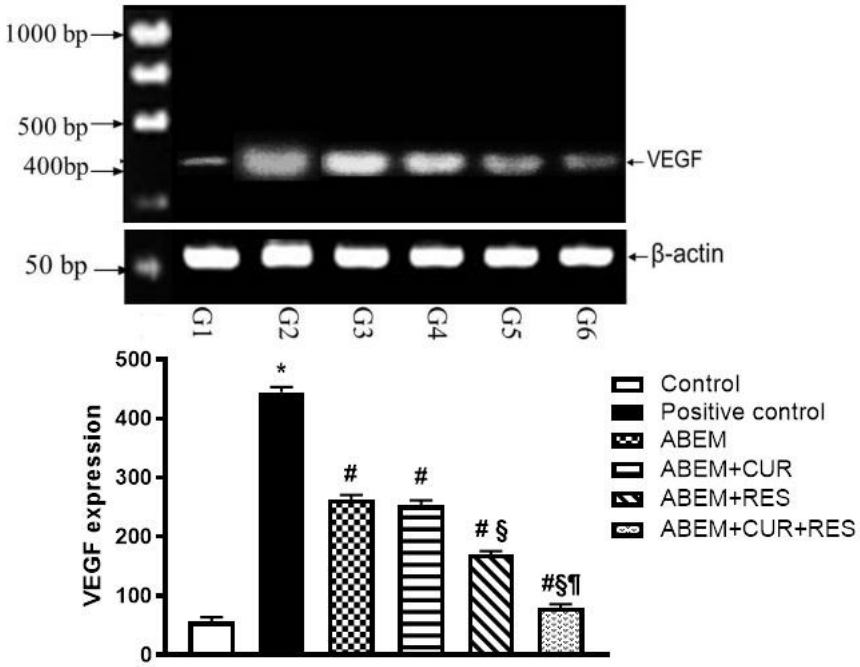

Figure 3: Effect of ABEM and its combination with CUR and/or RES on the expression of VEGF in the harvested cells. Data were expressed as mean±SEM.* Values differ significantly from negative control ($p < 0.05$).# Values differ significantly from positive control ($p < 0.05$).§ Values differ significantly from ABMA ($p < 0.05$).¶ Values differ significantly from ABMA & RES ($p < 0.05$).Statistical analysis was determined by one-way analysis of variance (ANOVA), followed by Tukey's test as a multiple comparison post-ANOVA test.

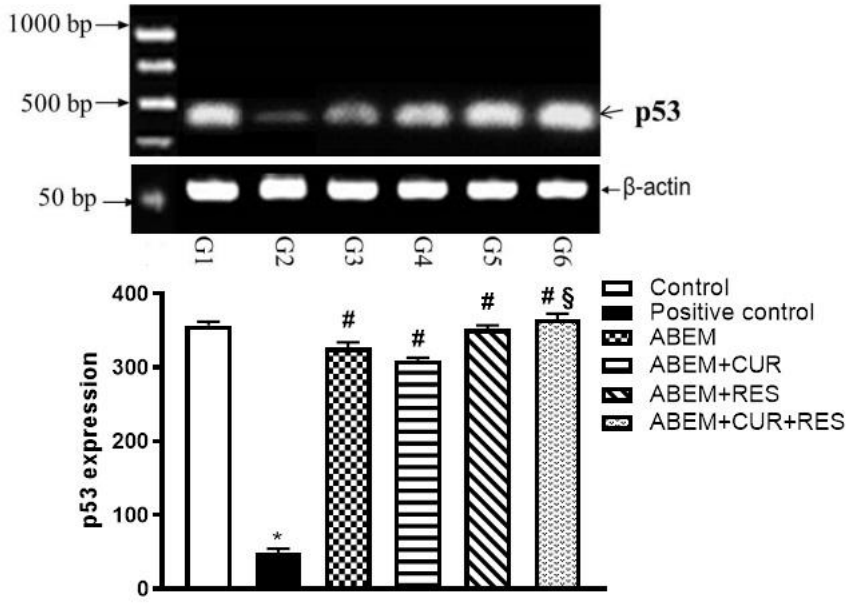

Figure 4: Effect of ABEM and its combination with CUR and/or RES on the expression of P^{53} in the harvested cells. Data were expressed as mean±SEM. * Values differ significantly from negative control ($p < 0.05$).# Values differ significantly from positive control ($p < 0.05$).§ Values differ significantly from ABMA ($p < 0.05$).¶ Values differ significantly from ABMA & RES ($p < 0.05$).Statistical analysis was determined by one-way analysis of variance (ANOVA), followed by Tukey's test as a multiple comparison post-ANOVA test.

Estimation of NF-κB1 and Caspase-3 levels:

The current study employed the ELISA technique to study the effects of combined drugs as anti-tumor agents, and to investigate the different signaling pathways. The results revealed that the level of NF-κB1 significantly increased in non-treated cell lines. On the other hand, treatment with ABEM and its combination with CUR or RES produced a significant reduction in the level of NF-κB1 with respect to the positive control group. The combination of ABEM with RES was more effective than ABEM alone. Incubation of MCF-7 cells with the combined three drugs significantly reduced NF-κB1 expression more than other groups (Fig 5). Meanwhile, a significant decreased level of caspase-3 was observed in non-treated cells. Treatment with drug combinations produced significant elevations in level of caspase-3 in treated groups with respect to non-treated groups. The combination group of ABMA with both CUR and RES produced the greatest protection effect (Fig 6).

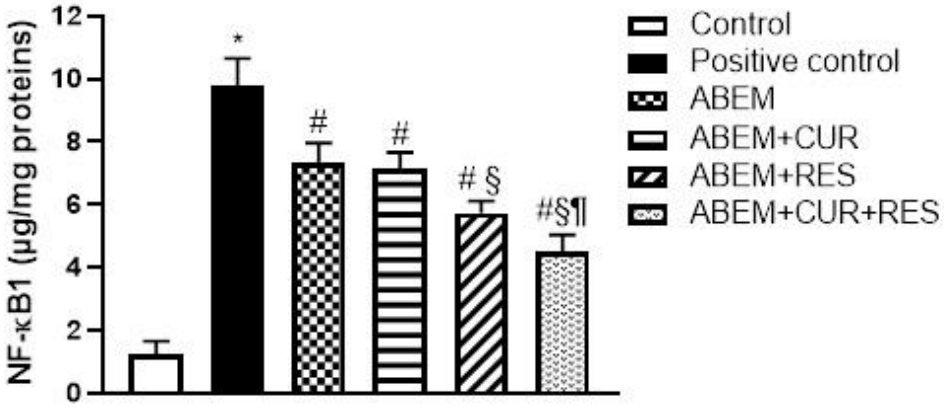

Figure 5: Effect of ABEM and its combination with CUR and/or RES on the level of NF-κB1 in the harvested cells. Data were expressed as mean±SEM. * Values differ significantly from negative control ($p < 0.05$).# Values differ significantly from positive control ($p < 0.05$).§ Values differ significantly from ABMA ($p < 0.05$).¶ Values differ significantly from ABMA & RES ($p < 0.05$).Statistical analysis was determined by one-way analysis of variance (ANOVA), followed by Tukey's test as a multiple comparison post-ANOVA test.

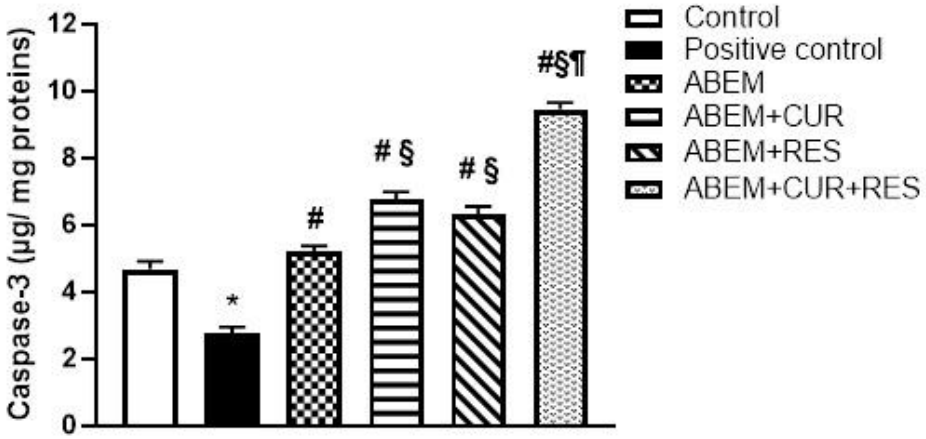

Figure 6: Effect of ABEM and its combination with CUR and/or RES on the level of Caspase-3 in the harvested cells. Data were expressed as mean±SEM. * Values differ significantly from negative control ($p < 0.05$).# Values differ significantly from positive control ($p < 0.05$).§ Values differ significantly from ABMA ($p < 0.05$).¶ Values differ significantly from ABMA & RES ($p < 0.05$).Statistical analysis was determined by one-way analysis of variance (ANOVA), followed by Tukey's test as a multiple comparison post-ANOVA test.

Estimation of MDA, NO and GSH contents and SOD activity:

The amount of GSH and SOD activity was significantly decreased in the intoxicated group compared to the healthy control group. Treatment with ABEM produced significant elevation in the content of GSH and SOD activity in comparison with the intoxicated group. After the incubation period with ABEM and its combination with CUR or RES, the level of GSH and SOD activity were significantly increased in all treated groups in comparison to ABEM alone. The greatest protection was found in the group treated with the

combination of ABEM, CUR and RES. Levels of MDA and NO were significantly increased in the positive control group compared to normal cells. In other groups after combined treatment with ABEM and/or CUR and RES, MDA and NO were significantly decreased with respect to the positive control group. The highest protection was observed in the group that was treated with ABMA, CUR, and RES (Table 2).

Table 2: Effect of ABEM and/or CUR and RES onP53, MDA, NF-κB1, NO and GSH contents and SOD activity in the harvested cells of breast cancer and control groups.

	Control	Positive control	ABEM	ABEM+CUR	ABEM+RES	ABEM+CUR+RES
P53 levels (ng/mg proteins)	2.3±0.13	0.93±0.04*	2.85±0.12#	3.26±0.17#	6.57±0.12#§	10.27±0.19#§¶
NF-κB1 levels (µg/mg proteins)	1.25±0.40	9.77±0.87*	7.31±0.64#	7.12±0.54#§	5.73±0.37#§	4.52±0.51#§¶
MDA (ng/ mg proteins)	1.57±0.11	12.5±0.23*	8.89±0.15#	9.50±0.12#	5.44±0.15#§	4.06±0.14#§¶
NO (µM/g proteins)	22.76±1.25	47.65±1.1*	38.73±1.3#	41.57±1.31#§	36.05±1.24#§	29.75±1.18#§¶
GSH (µg/g proteins)	12.13±1.02	2.47±0.12*	4.49±0.31#	4.18±0.33#	7.46±0.39#§	9.88±0.54#§¶
SOD (U/mg protein)	19.55±0.43	5.13±0.39*	11.3±0.44#	9.31±0.47#§	15.21±0.46#§	18.33±0.47#§¶

Data were expressed as mean±SEM. MDA: malondialdehyde, NO: nitric oxide, GSH: reduced glutathion and SOD: super oxide dismutase.
* Values differ significantly from negative control ($p < 0.05$).
# Values differ significantly from positive control ($p < 0.05$).
§ Values differ significantly from ABMA ($p < 0.05$).
¶ Values differ significantly from ABMA & RES ($p < 0.05$).
Statistical analysis was determined by one-way analysis of variance (ANOVA) followed by Tukey's test as a multiple comparison post-ANOVA test.

IV. Discussion

Breast cancer is one of the most frequent invasive cancers diagnosed among women worldwide. Breast cancer is a distinct disease that is recognized by the discrimination and reproduction of cancer cells that have abnormalities which express several genes.(Harirchi et al., 2010) Chemotherapy is still a wonderful treatment for numerous types of cancers, however, some patients suffer from its dangerous side effects and toxicity. ABEM is one of the most effective chemotherapies available for patients with breast cancer and is the first selective inhibitor of CDK4 and CDK6. ABEM is a chemotherapeutic drug which permits continual dosing to obtain the sustained inhibition of breast cancer.(Patnaik et al., 2016) A combination of chemotherapy and natural products may have a synergistic effect against different types of cancers. This combination may also help in reducing the toxicity associated with chemotherapy. In the present study, the combination of CUR and RES with ABEM was investigated for their synergistic effect. We were able to show that this combination inhibited the proliferation of breast cancer cells in the breast cancer cell line MCF-7. Our results are consistent with the findings of many previous studies reporting the broad biological activities of natural compounds including immunity enhancement, antiviral, anti-inflammatory, antioxidant and even anti-tumor effects.(Miyata, 2007)

In the present study, the CI values were calculated (CI<1), indicating a synergistic effect between ABEM, CUR, and RES against breast cancer. The development of a safe treatment for breast cancer with ABEM by the addition of the safe phytochemicals as CUR and RES was the main target of the current study. Thus, the present study endeavored to discuss the anti-tumor effects of CUR and RES and the basic mechanisms of these phytochemical combinations with ABEM on breast cancer cells with the aim of increasing its efficacy and lowering the dose of ABEM, and hence its adverse effects.

In the present study, the expression level of miR-4270, which is considered a biomarker of breast cancer(Hamam et al., 2016)'(Babaei et al., 2018), was estimated using RT-PCR. Levels of miR-4270 were found

to increase in breast cancer cells (positive control group), but its level was reduced following treatment with CUR and RES (group IV). Moreover, miR-4270 expression was markedly decreased following treatment with the combination of ABEM, CUR and RES (group VI). Our results are in line with a previous report indicating the increase of the expression level of miR-4270 in the blood of breast cancer patients.(Hamam et al., 2016)

There are several possible molecular pathways that can be used to interpret the synergistic anti-tumor effects of CUR and RES with ABEM. The present study revealed that CUR and RES possess apoptotic, anti-proliferative and antioxidant effects by the measurement of several parameters, such as VEGF, p53, NF-κB1, Caspase-3, NO, MDA, GSH and SOD. Many natural products have diverse biological activities including anti-inflammatory, antioxidant and even anti-tumor effects(Aggarwal et al., 2013). Indeed, CUR was announced previously to produce its activity through different pathways.(Wang et al., 2016) Moreover, RES has also been shown previously to exhibit different biological effects, such as anti-inflammatory effects, cardio-protective effects, and anti-tumor activities.(Cui et al., 2010)

Our results demonstrated that p53 levels were increased following the treatment with the combination of ABEM, CUR, and RES. These results reported the possible anti-proliferative effect of CUR and RES, and pointed to the improvement of the efficacy of ABEM. Recently, several studies showed that CUR encouraged apoptosis in breast cancer cells by increasing the activity of p53, which increases Bax, and hence increasing the Bax/Bcl-2 proportion(Choudhuri et al., 2002).

Estimation of VEGF levels showed that the combination betweenCUR and RES potentiates the anti-angiogenic effect of ABEM. VEGF levels in breast cancer cells have increased significantly compared with the control of healthy normal cells. Levels of VEGF were markedly decreased following the treatment with ABEM and the combination of CUR and RES. Our results are in harmony with a previous study which reported that the VEGF enhances the development and metastasis of breast cancer.(Luo et al., 2016)

ABEM discouraged the NF-κB1 pathway, which was evident by the reduction in NF-κB1 levels following treatment. This effect was potentiated by CUR and RES. This result is in line with a previous report which indicated the role for NF-κB1 in cancer suppression mechanism.(Qie and Diehl, 2016)

To investigate the apoptotic and anti-tumor activity of ABEM with CUR and RES, caspase-3 levels were estimated. Levels of caspase-3 were markedly increased upon the treatment withthe combination therapy used in the present study. Previous studies showed impaired apoptosis to be a hallmark of cancer.(Rogers et al., 2019)

The current research investigated the effect of CUR and RES on antioxidantthrough the estimation of NO, MDA and other antioxidants. Levels of NO and MDA were dramatically decreased in different therapeutic combination groups. In the same manner, levels of antioxidants such as GSH and SOD were increased significantly in the treated group with ABEM, CUR and RES. In accordance with our results, several lines of evidence indicated a marked decrease in MDA levels produced by chemotherapy and anti-inflammatory drugs.(Ekor et al., 2013)

V. Conclusion

The current study provides evidence that the combination of ABEM with CUR and RES produces a synergistic effect in the cells of breast cancer, as demonstrated by several molecular pathways including apoptotic, angiogenesis inhibition and antioxidant effects. We were able to demonstrate that the proposed combination of ABEM, CUR, and RES increases the efficacy of ABEM, which may help to reduce its dose of ABEM and, therefore, adverse effects.

Conflicts of interest

The authors declare that this article has no conflicts of interest.

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Authors' contributions

TO and AW create idea, conceived and designed research. TO and MAK performed RT-PCR and ELISA techniques. AW and MAK analyzed and interpreted the results. TO and MAK write manuscript draft. TO is the major contributor in editing and writing the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests

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