Journal of Environmental Sciences

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Reprint

Volume 53, Number 2: 21-35

(2024)

http://Joese.mans.edu.eg **P-ISSN 1110-192X**

e-ISSN 2090-9233

Journal of Environmental Sciences

JOESE 5

ISSN 2090-9233

Journal homepage http://joese.journals.ekb.eg

Original Article

Enhancement of Radiation therapy for Breast Cancer through Combined Treatment with Indole-3-Carbinol Aggregation with Resveratrol: Assessment of EMT Biomarkers and Effects on Cell Proliferation

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Article Info

Article history:

Received 11/ 01 /2024

Received in revised

form 1/03/2024

Accepted 07/04/2024

Keywords: *Breast Cancer, Indole-3-Carbinol, Resveratrol, EMT, Juncus, ionizing radiation.*

Abstract

Breast cancer is a heterogenous group of malignant tumors which is the most common cancer that affected women worldwide. Despite radiotherapy considered as one of the most major strategies for cancer treatment, but it is still limited due to the developed resistance by highly metastatic cancers. This study is designed to assessment the anti-cancer characteristics and radio-sensitizing effect of Indole-3-carbinol (I3C) and Resveratrol (RES) on MDA-MB-231 cells. the effective dose of I3C and RES that will be used as a radio-sensitizing dose was assessed using MTT assay. Treatment of MDA-MB-231 cells with I3C+ RES, and/or ionizing radiation (IR) led to significant downregulation at cell proliferation as indicated by the decreased of developed colony ratio. The combined therapy induced apoptosis by which was indicated by the significant increase of Bax and downregulation of Bcl-2 level expression. Combined treatment significantly attenuated MDA-MB-231 cells motility which was validated using wound healing assay after 24hr of pretreatment. Combined treatment revealed a significant reduction at Nrf2 level which in turn leads to downregulation at both anti-oxidant enzymes Catalase and Glutathione Synthesis. the pretreatment with I3C+ RES, and/or IR revealed a significant decrease on e-cadherin, and elevation at vimentin level expression. This study assesses the anti-cancer effect of I3C plus RES, by its important role in spectacular cell responsiveness to IR leading to a significant inhibition at MDA-MB-231 cell proliferation, migration and survival.

1. Introduction

Breast cancer (BC) is a heterogeneous group of tumors, has taken the place of lung cancer **(He et al., 2023),** which is the most common cancer affecting the females and increased with age. BC is one of the most serious causes of death in females worldwide **(Wang et al. 2023; Schonberg et al., 2023; Rakha et al., 2023),** and the molecular biology of its are still unclearly **(Jayachandran et al., 2023).** Many reports revealed that about 2-2.5 million cases are diagnostic globally per year **(Hussain et al., 2023),** and 520,000 new cases per year are detected in Europe, about 90% of its are at early stages **(Pruneri et al., 2023).** Despite most breast cancer is discontinuous, about 5-10% are hereditary **(Sokolova et al., 2023).** In the low- and middle-income countries, estimated that 5 million women have a history of breast cancer and underlying at risk of treatment for long-term toxicity **(Sarrade et al., 2023).** However, the breast cancer current-treatments were fit limited like surgery, chemotherapy and radiotherapy, with a high rate of side effects (Wang et al. 2023). Adjuvant radiotherapy has an important role in breast cancer management as a standard approach for early stages

due the benefits of improving locoregional recurrence rates and reducing cancer-related mortality (He et al., 2023; Franco et al., 2023). Radiotherapy is one of many strategies that used for cancer treatment due to its high energy that causes DNA double strand breakage or through oxidative stress by initiate free radicals in cancer cells (Nosrati et al., 2023). The physical ionizing radiation (IR) (X-ray and gamma ray) are the most common rays that used to kill tumor cells (Ghazy et al., 2023). the high energy of its are used to deliver the irradiation doses in tumor regions for cancer treatment. However, radiotherapeutic is mannered by its low radiosensitivity, inaccurate tumor localization, and lack differentiation between lesions and the revers effect of irradiation in the normal tissues (Ashrafizadeh et al., 2023; Nosrati et al., 2022; Wei et al., 2022). A high level of oxidative stress is considered a novel target for anti-cancer cure due to increasing of exogenous reactive oxygen species (ROS) and/or anti-oxidant system inhibition (Loenhout et al.,2020; Galadari et al., 2017). Excessive amounts of ROS may act as cellular toxicants, which can lead to cancer-cell growth arrest, apoptosis, and necrosis. It is speculated that malignant cells under increased levels of oxidative stress are more vulnerable to further ROS attacks (Mileo et al., 2016). The radiotherapy strategy is based on IR, which increases ROS generation and induces apoptotic damage in cancer cells (Kim et al., 2019). There is a widespread consensus that radio-sensitization efforts should prioritize the integration of phytochemicals and radiation. Therefore, it is major to look for an alternative strategy to higher radiosensitivity of tumors while simultaneously lower their systematic revers effects.

In last decade, therapeutic effectiveness of phytochemicals has been frequently used as a potential cancer treatment technique in theranostics application (Jit et al., 2022). Phytochemicals are a natural extraction of plants (fruits, vegetables, leaves, herbs and roots) that has a high potent against cancer cells, and characterized as anti-tumor, anti-angiogenesis, anti-inflammatory and antioxidant (Shree et al., 2019). The effectiveness of phytochemical combinations in cancer therapy may be due to their capacity to downregulate anti-apoptotic proteins pathways and to upregulate the apoptotic proteins pathways that induce cell death, restrict cell proliferation and invasion, sensitize malignant cells, and enhance the immune system (Shafiq et al., 2022; Zheng et al., 2022).

Indole-3-carbinol (I3C) is a natural plant extraction that derived from metabolize of glucobrassicin A, and widely found in cruciferous vegetables like cabbage, kale, sprouts, broccoli, etc. (Wang et al., 2022; Qi et al., 2022). Several studies elucidated the I3C importance role in cancer cells, indol-3-carbinol promoted cancer cell apoptosis and inhibited angiogenesis by diminishing the activity of the Akt/NFκB signaling (Qi et al., 2022; Abdraboh et al., 2020). Some of literatures showed that I3C playing an important role in suppressed proliferation and induced cell apoptosis when tested on breast cancer, ovarian cancer, hepatocellular carcinoma, and prostate cancer (Martín-Ruiz et al., 2018). numerous studies have proved that I3C is involved in inducing apoptosis and inhibiting cell cycle progression and tumor proliferation (Lee et al., 2019; Choi et al., 2017).

Resveratrol (RES) (3,4,5-transtrihydroxystilbene) is a phytoalexin that derived from phenylpropanoids and extracted from grapes, wine, pomegranate, pines and peanuts (Talib et al., 2022; Komorowska et al., 2021). Recently, resveratrol playing important role in increased capacity to decrease the proliferation of highly metastatic (MDA-MB-231) and less aggressive metastatic (MCF-7) breast cancer cells (Lacerda-Abreu et al., 2021; Rodríguez-Enríquez et al., 2019). Resveratrol treatment of breast cancer inhibits cell migration by decreasing matrix metalloproteinase-2 (MMP-2), MMP-9, p-phosphoinositide 3 kinase (p-PI3K), p-AKT, Vimentin, Snail1, and Slug levels (Lacerda-Abreu et al., 2021; Sun et al., 2019). Resveratrol is also reported to promote metabolism and reduce oxidative stress, which can also be used as an antioxidant affecting the synthesis of nitric oxide that regulates DNA damage, cell cycle, apoptosis, and proliferation (Dembic et al., 2019; Wu et al., 2019).

In this present study, we elucidated the therapeutic effect of I3C and RES aggregation as co-sensitizing factor, combining radiation in sensitizing MDA-MB-231 cells through inhibiting cell proliferation, motility and survival.

2. Material and Methods.

2.1. Chemicals and Antibodies

MDA-MB-231 cells, kindly provided by Tissue culture lab of Zoology dept., faculty of Science/El-Mansoura university, El-Mansoura, Egypt., were maintained in Dulbecco's Modified Eagle Medium (DMEM) (biowest, Canada), richen with 10 % Fetal Bovine Serum and penicillin/streptomycin (biowest, Canada, trypsin-EDTA 1X in solution w/o calcium, w/o magnesium, and w/Phenol Red). The cells were incubated at 37 ◦C and 5% CO2. Cell harvest was conducted by washing cells twice with 1x Phosphate

Buffer Saline (PBS) followed by trypsinization using 0.25 % Trypsin at 5 % CO2 and 37 ◦C (biowest, Canada). phytochemicals "resveratrol and indole-3-carbinol" that was purchased from MedChemExpress (MCE), USA. The antibodies (Bax, Bcl-2, nuclear factor erythroid 2 (Nrf2), E-cadherin (E-cad), Vimentin (Vim) and GAPDH) were purchased from (Santa Cruz, CA, USA).

2.2. Cell line and cell culture

2.2.1.MTT assay (proliferation assay)

MDA-MB-231 cells were plated in 96-well plate with 200 cells per well with 300µl of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in triplicate for each concentration and incubation for 24hr. Different concentrations of RES and I3C were prepared by serial dilution. Descending concentrations of RES and I3C (200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, and 6.25µg/ml) were transferred to the cells in the 96- well plate, included untreated cells as control with 0.1% DMSO and was incubated for 48hr. After incubation, the IC50 of RES and I3C and the inhibition at cell proliferation was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, 5 mg/ml), 100µl MTT was added to each well and incubated for 4hr at 37ºC. After incubation, SDS (sodium lauryl sulfate) 100µl was added to each well then incubated for 14hr. The absorbance was measured at a wavelength of 570 nm using (Bio Tek, Elx800, US) (Wilsher et al., 2017). The inhibition percentage in cell proliferation was calculated from (Eq.):

$Inhibition\ (\%) = Control\ (OD) - Sample\ (OD) / Control\ (OD)$ X 100 % , Where, (O.D) is the optical density.

2.2.2. Group designation

To estimate the phytochemicals therapeutic potential in combining with/without IR irradiation on MDA-MB-231 cells treatment, MDA-MB-231 cells were divided into 4 groups as followed: 1) untreated MDA-MB-231 cells (control), 2) RES + I3C group (cells treated with 28.53 µg/ml of RES + 58.82 µg/ml of I3C), 3) IR group (cells treated with single dose of 2Gy x-ray), 4) RES + I3C + IR group (cells treated with 28.53 µg/ml of RES + 58.82 µg/ml of I3C + single dose of 2Gy x-ray). These four groups were elucidated the impacts of radio-phytochemical for 24hr pre-treatment against MDA-MB-231 cells by colony formation, anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax, and the cells migration ability through wound healing assay. Besides, this groups were tested to clarify the therapeutic potential of I3C + RES and/or IR through valued cell migration, endothelial-mesenchymal transition genes expression (E-cadherin (E-cad) and vimentin (Vim)), and oxidative stress by tested anti-oxidant enzymes Catalase (CAT) and Glutathione Synthesis (GSH) on MDA-MB-231 cells. The untreated MBA-MB-231 cells group was considered as a control group.

2.2.3. Clonogenic assay

MDA-MB-231 cells were sub-cultured in T-75 flasks, two flasks for each group, and incubated till confluence of about75% - 80% then pre-treated with RES + I3C for 24 hours. MDA-MB-231 cells were irradiated with 2Gy x-ray, using a SHINVA® XHA600D medical linear accelerator (linac) and Prowess® panther 3D planning system as a radiation source. After irradiated, flasks of each group were washed twice with 1x PBS and trypsinization. MDA-MB-231 treated cell was seeded in triplicate 6well plate with 1x103 cells per well. After incubation for an additional 7 days until the survived cells develop colonies countable by naked eye. Cells were fixed with 70% ice-cold ethanol and then stained with crystal violet. Colonies of at least 50 cells were counted. Survival factor and colonies formation ratio were estimated for each group (**Abdelrazzak, 2010).**

2.2.4. Wounded healing assay (motility assay)

MDA-MB-231 cells were soiled in T-75 flasks (two flasks for each group) and incubated till 75% - 80% confluence then pretreated with RES + I3C for 24 hours and irradiated. MDA-MB-231 cells were washed twice with 1x PBS and trypsinization, MDA-MB-231 treated cells were seeded in 6well plate with 2.5x104 cells per well then incubated even completely confluence, A scratch with 1000µL micro-pipette tip was induced diagonally in the middle of each plate. Afterwards, cells were washed twice with PBS to remove the undetached cells and then incubated for 24hr. Microscopic photographs were taken using inverted microscope at 0 and 20hr **(Pandya et al., 2020).**

2.3 Real-Time Polymerase Chain Reaction (RT-PCR) for evaluation of Bax, Bcl2, Nrf2 e-cadherin, and vimentin transcriptional levels

Total RNA was extracted from MDA-MB-231 cells using Trizol method according to manufacturer's instructions. The quality and concentration of total extracted RNA were detected by (Nano Drop ND-2000) spectrophotometer (Thermo Fisher, DE, USA). Complementary DNA was synthesized from 1 µg of total RNA using

SensiFAST™ cDNA Synthesis Kit (Bioline, Australia) according to the manufacturer's protocol. Determination of the expression levels were assessed using Maxima SYBR Green Kit according to manufacturer's protocol (Thermo Scientific, CA, USA) using the following primers: Bax (sense)5′-GGTTGTCGCCCTTTTCTA-3′, (antisense) 5′-CGGAGGAAGTCCAATGTC-3′, Bcl2: (sense)5′-GATGTGATGCCTCTGCGAAG-3′, (antisense) 5′-CATGCTGATGTCTCTGGAATCT-3′, CD44: (sense)5′-TTTGCATTGCAGTCAACAGTC-3′, (antisense) 5′-TTACACCCCAATCTTCATGTCCAC-3′, and GAPDH: (sense) 5′-TGGCACCCCAGCACAATGAA-3′, (antisense) 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′. Thermal cycling conditions were conducted by initial heating step at 95 ◦C for 10 min. followed by 40 cycles at 95 ◦C for 30 s, annealing at 49 ◦C, 64 ◦C and 51 ◦C for Bax, Bcl-2, and GAPDH, respectively, for 30 s each and 72 ◦C for 1 min and final extension at 72 ◦C for 10 s. Relative expression of Bcl-2 and Bax mRNA was normalized against GAPDH transcript as an internal reference control then calculated according to mathematical model introduced by M Pfaffl 2-ΔΔCt (Golestani et al., 2014).

2.4 Assessment of oxidative stress biomarkers

MDA-MB-231 cells were treated with I3C + RES for 24hr and irradiated with 2Gy x-ray then incubated for an hour at 37ºC and 5% of Co2 till harvested, cells were washed with 1x PBS and trypsinization then kept with 1ml of 1x PBS in -20 ºC till use. ELISA technique was used to determinate the Catalase (CAT) and Glutathione Synthesis (GSH) antioxidants according to the kit instructions (Biodiagnostic and research reagents, Giza, Egypt).

2.5 Statistical analysis

The data was statistically analyzed using a statistical software GraphPad Prism program (V.6). mean value and standard deviation were calculated. The mean differences were acquired by One-way ANOVA analysis. The final results were expressed as mean± standard error of the mean value (Mean ± SEM) and used at least 3 values for the samples (n= 3).

3. Results

3.1 Determination of I3C and RES IC_{50s}

I3C and RES were first indicated by MTT colorimetric method which is the useful tool for assessing cell proliferation, cytotoxicity, and cellular metabolic activity against the log of [I3C, and RES doses individually] (fig. 1A and B). the data analysis indicated 200µg/mL as the calculated IC50 dose of I3C and RES cytotoxic effect on MDA-MB-231 cells. Despite of, the dose of 58.82µg/mL and 28.53µg/mL, 2Gy x-ray for I3C, RES, and IR (ionizing radiation), respectively, were chosen for radio-sensitizing studies.

3.2 Effect of I3C + RES with/without IR on MDA-MB-231 cells proliferation

3.2.1 Effect of I3C + RES with/without IR on MDA-MB-231 cells colony formation ratio

To estimate the radio-sensitizing effect of I3C and RES with/without IR, MDA-MB-231 cells were treated in presence and absence of 58.82µg/mL and 28.53µg/mL, of I3C and RES, respectively, for 24hr then irradiated with single dose of 2Gy of X-rays, due to its relevance to the cumulative dose used in human conventional radiotherapy as a routine protocol, and it also showed a significant effect on clonogenic assay. The number of developed colonies was assessed after 7 days of incubation. the data showed a significant reduction in the number of colonies formation in all treated groups I3C + RES, and IR by (66.33 ± 3.75, 304.66 ± 5.17), as compared to control group (408.33 ± 4.41) $p<0.05$, respectively. Remarkable, data showed a synergistic effect in combined therapy in MDA-MB-231 cells by (1.33 ± 0.33), when cells treated with I3C + RES + IR as compared to control group (408.33 ± 4.41), respectively (fig. 1C, D). the significant decrease of developed colonies ratio for I3C + RES treatment compared with control group pointed out the anti-growth and anti-survival effect of combination on MDA-MB-231 cells.

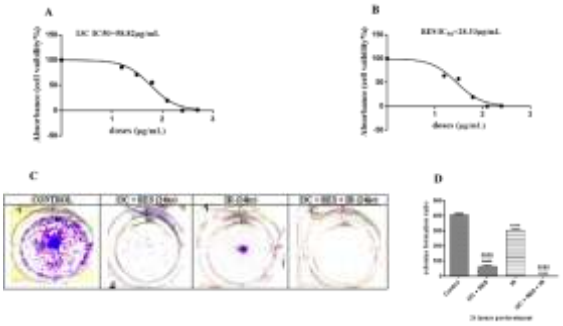

Figure (1): Determination of I3C IC50 dose effect on MDA-MB-231 cells after 2 days of treatment presented by Exponential curve of MTT assay (A). Determination of RES IC50 dose effect on MDA-MB-231 cells after 2 days of treatment presented by Exponential curve of MTT assay (B). effect of I3C + RES, and/or IR on MDA-MB-231 at clonogenic assay (C). Radio-sensitizing effect of I3C + RES, and/or IR on cell survival. The 24hr pre-sensitized MDA-MB-231 cells were exposed to IR, Combined therapy of I3C + RES + IR significantly inhibited the colony formation ratio compared to untreated control cells (D).

The data of experiment, were expressed as mean of three separate experiments ± SEM. Level of significance was denoted as follows: *, **, ***, **** $p < 0.05$, as compared to control cells. $, $$, $$$, $$$$ $p<0.05$, as compared to IR treatment group.

3.3 I3C + RES, and/or IR treatment induce cell apoptosis

The apoptotic analysis was verified by RT-PCR technique that performed to determine the anti-apoptotic protein bcl-2 and pro-apoptotic protein Bax in MDA-MB-231 cells, as the induction of apoptosis by downregulate bcl-2 gene expression and upregulate Bax gene expression. The analyzed data revealed significant increase in the level of Bax gene expression when MDA-MB-231 cells treated with I3C + RES, IR, and I3C + RES + IR recorded (4.33 ± 0.066, 1.23 ± 0.012, 1.44 ± 0.015) 24hr of treatment as compared to control group (1 ± 0.058), $p<0.05$, respectively, (fig 2A).

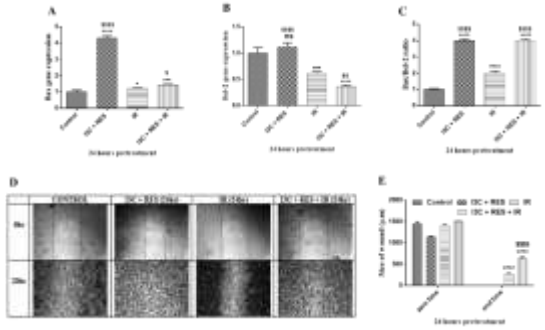

Figure (2): Apoptotic analysis detected by RT-PCR technique, indicated a significant upregulation of Bax gene expression levels 24hr pre-treatment on MDA-MB-231 cells in combined with IR (A). Bcl-2 gene expression levels significant attenuation after 24hr pre-treatment (B). Bax/Bcl-2 ratio indicated significant elevation of Bax gene expression against Bcl-2 levels of expression (C). Effect of I3C+ RES and/or IR on MDA-MB-231 cell motility after 24hr pre-treatment (D). Wound healing migration assay. The size of induced wounds was monitored over 20hr till closure of the wound of control untreated cells. Statistical analysis of the wound size indicated the significant inhibitory effect of IR, and I3C + RES + IR after 24hr treatment on cell migration capability, respectively (E). The data of experiment, were expressed as mean of three separate experiments ± SEM. Level of significance was denoted as follows: *, **, ***, **** $p < 0.05$, as compared to control cells. $, $$, $$$, $$$$ $p<0.05$, as compared to IR treatment group.

The data illustrated a non-significant increase of Bcl-2 expression levels on MDA-MB-231 cells treated with I3C + RES by (1.118 ± 0.043) $p<0.05$, while groups treated with IR, and I3C + RES + IR revealed significant decrease of Bcl-2 expression levels by (0.62 ± 0.012, 0.36 ± 0.015) after 24hr of treatment as compared to control group (1 ± 0.058), $p<0.05$, respectively (fig. 2B).

Interestingly, the index of Bax/Bcl-2 ratio data confirmed the apoptotic therapeutic effect of I3C + RES and/or IR on MDA-MB-231 cells, the data showed a significant elevation at the levels of expression of Bax against Bcl-2 on MDA-MB-231 cells treated with I3C + RES, IR, and I3C + RES + IR for 24hr of treatment recording (4.00 ± 0.058, 2.017 ± 0.044, 4 ± 0.046) as a compared to untreated cells group (1 ± 0.058), $p<0.05$ (fig. 2C).

3.4 Effect of I3C + RES, and/or IR on MDA-MB-231 cell motility (wound healing assay)

The effect of I3C + RES, with/without IR on MDA-MB-231 cells motility was tested by measuring the migration ability of treated cells to migrate following mechanically induced wound in the culture plate; whereas the increase at wound size illustrated an inhibition at cell migration capability. Estimation of the size of the empty space after 24hr of treatment revealed a significant effect of IR, and combined treatment by (270.66 ± 12.979, 628.66 ± 21.48, $p<0.05$) as a compared to control group (0 ± 0.0), respectively, in lessening the MDA-MB-231 cells motility compared with control cells which migrated into the empty space and healed the induced wound after 20hr of starting the experiment (fig. 2D and F).

3.5 I3C + RES and/or IR effect on the antioxidant machinery of MDA-MB-231

MDA-MB-231 cells were exposure to I3C + RES for 24hr and/or IR brought about significant increase at Nrf2 gene expression in all groups treated with I3C plus RES, IR, and combined treatment recorded (5.27 ± 0.145, 8.68 ± 0.03, 3.17 ± 0.015), respectively, as compared to control group (1 ± 0.058), remarkable, the data reached significant reduction at Nrf2 expression when cells treated with I3C plus RES and combined treatment as compared with IR treated cells (fig. 3A).

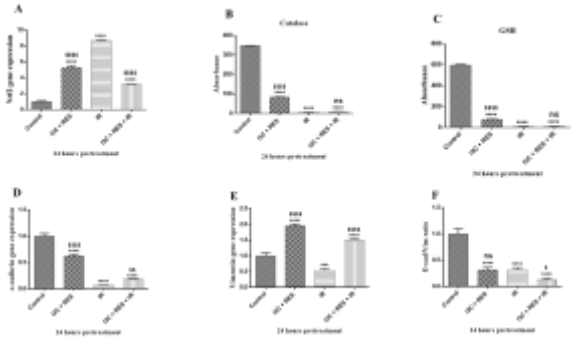

Figure (3): Effect of I3C+ RES and/or IR on Nrf2 expression on MDA-MB-231 cells 24hr pretreated revealed a significant increase on Nrf2 levels as compared to control group, and significant decrease on Nrf2 expression as compare to IR treated cells (A). anti-oxidant CAT levels detected by ELISA technique revealed a significant decrease on MDA-MB-231 cells after 24hr pre-treated in all treated groups (B). GSH ELISA data showed a significant decrease on MDA-MB-231 cells after 24hr pre-treated with I3C + RES, IR, and combined treatment (C). E-

cadherin expression on MDA-MB-231 cells 24hr pretreated with I3C + RES, and /or IR revealed a significant decrease at E-cad levels in I3C + RES, IR and combined treatment (D). vimentin expression on MDA-MB-231 cells 24hr pretreated with I3C + RES, and /or IR revealed a significant increase on Vim levels in I3C + RES, and combined treatment groups, with significant decrease at Vim level on cells treated with IR (E). E-cad/Vim ratio indicated significant reduction of E-cad gene expression against Vim levels of expression (F). The data of experiment, were expressed as mean of three separate experiments ± SEM. Level of significance was denoted as follows: *, **, ***, **** $p < 0.05$, as compared to control cells. $, $$, $$$, $$$$ $p<0.05$, as compared to IR treatment group.

Furthermore, the data showed significant decreased at the antioxidant enzyme CAT when treated with I3C + RES, IR and combined treatment by recorded (84.014 ± 0.78, 7.615 ± 0.582, 9.69 ± 0.49), $p<0.05$, respectively, as compared with untreated control group (348 ± 0.217) (fig. 3B). Besides, the activity of GSH enzyme showed a significant decrease when MDA-MB-231 cells treated with I3C + RES, IR, and combined treatment which recorded (78.23 ± 0.96, 7.069 ± 0.67, 11.11 ± 0.69), $p<0.05$, respectively, as compared to control group (596.329 ± 3) (fig. 3C).

3.6 Effect of I3C plus RES and/or IR on EMT on MDA-MB-231 cells

The effect of I3C + RES with/without IR was tested by RT-PCR technique, the collected data observed that MDA-MB-231 cells exposure to I3C + RES, IR, and combined treatment significantly decreased the expression of E-cad recorded (0.625 ± 0.015, 0.065 ± 0.003, 0.185 ± 0.005) $p<0.05$, respectively, as compared to control group (1 ± 0.058) (fig. 3D). Besides, the treatment with I3C plus RES and combined treatment significantly elevation the expression of E-cad levels as compared with IR treated cells. Meanwhile, the I3C plus RES and/or IR pretreatment of MDA-MB-231 cells showed significant increased on Vim gene levels expression when treated with I3C + RES, and combined treatment by (1.96 ± 0.027, 1.493 ± 0.025), $p<0.05$, respectively, as compared to control group, while MDA-MB-231 cells treated with IR marked a significant decreased on Vim levels recorded (0.543 ± 0.029), compared to control group (1 ± 0.058) (fig. 3E). Observable, the treatment with I3C plus IR and combined treatment revealed a significant increase on Vim levels as compared to IR treated cells.

On the other hand, the index data of E-cad/Vim ratio on MDA-MB-231 cells treated with I3C + RES, and/or IR revealed significant increase on vimentin expression on groups treated with I3C + RES, IR and combined treatment by (0.315 ± 0.026, 0.318 ± 0.02, 0.127 ± 0.018), $p<0.05$, respectively, as compared to control group (1 ± 0.087) (fig. 3F).

4. Discussion

Radiotherapy is one of the major strategies for cancer diseases treatment. Despite of its ability to break down double strand DNA, the effectiveness of its still limited due to the ability of developed resistance by highly metastatic cancers that considered an obstacle for radiologist and oncologist (Abdraboh et al., 2020). several literatures elucidated characterizations of phytochemicals such as anti-cancer, anti-oxidant, anti-inflammatory, and anti-angiogenesis activities, the applied of its from laboratory to clinical trials protocol still limited due to its low bioavailable levels (Kotecha et al, 2016). Along decade, phytochemicals were used to enhancement cancer cells radiotherapeutic treatment by targeting many signaling pathways that support hypothesis to reduce breast cancer cells and cancer stem cells proliferation, survival and metastasis (Chiou et al., 2018; Chu et al., 2021). Relying to these works, I3C plus RES were combined to sensitizing breast cancer cells for radiotherapy.

In this present study, we aimed to assess the therapeutic potential of I3C plus RES as a radio-sensitizers, to enhance MDA-MB-231 breast cancer cell for responsiveness by reducing their proliferation, survival and migration. Additionally, the anti-cancer and anti-oxidant roles of I3C plus RES against MDA-MB-231 cancer cells were tested well.

In order to examine the anti-cancer role and the possible radio-sensitizing effect of I3C and RES, the IC50 of both were first calculated and a dose of (58.82 µg/ml, 28.53 µg/ml, respectively) were chosen for further radio-sensitizing studies as it were the doses that induced a significant inhibition at cell proliferation with enough account of cells remains for clonogenic assay after radiation. The significance of combined treatment compared to the control group indicated the radio-sensitizing effect of I3C plus RES in sensitizing up the cell to IR (fig 1A-C). the significant downregulate at the developed colony ratio in combined treatment and other groups compared to control group elucidated the collective effect of using I3C plus RES with IR on inhibiting cell growth (fig 1D). The important role of phytochemicals such as I3C and RES as a supplementary approach to classical chemo- and radio- therapies was confirmed by several studies (Chu et al., 2021; Chiou et al., 2018). A study by Zammanian et al., proved that combined treatment of glioblastoma U87MG cancer cell line with ordinal 2 Gy IR dose and genistein (50µM) revealed

the highest significant inhibition on cells proliferation and clonogenicity compared to 2 Gy IR and fractionated doses (Zammanain et al., 2016).

Moreover, the pro-apoptotic role of phytochemicals was tested through Bax and Bcl-2 genes expression. The significant upregulation of Bax level expression and downregulation of Bcl-2 level expression in combined therapy in comparison with either treated groups or control group indicated a typical p53-dependent pathway targeted by I3C + RES and/or IR (fig 2A-C).

Several studies proved the pro-apoptotic effect of phytochemicals as an adjuvant approach of classical therapies (chemo- and radio-therapies) in sensitizing cancer cells for radiotherapy by targeting P53/Bcl-2 pathway (Enomoto et al. 2017; Wang et al., 2015).

Furthermore, the metastatic inhibition effect of I3C + RES and/or IR, compared to untreated cells was revealed by the observed inhibition at cell motility in wound healing assay (fig. 2D and E). interestingly, the combined therapy compared to control group showed a significant inhibition effect of cell motility to close the empty space of the scratched wound due to dispreading MDA-MB-231 cells proliferation (fig. 2D and E).

On the other hand, the antioxidant effect of I3C plus RES and/or IR has been proven by decreased the levels of Nrf2 which playing important role in regulating the activity of antioxidant enzymes CAT and GSH (fig. 3A-C). One of the defensive mechanisms that cancer cell used to survive was to upregulated Nrf2 expression in order to upraise the antioxidant enzymes expression to lower the level of oxidative stress. Adjuvant therapy with combined treatment, significantly downregulated Nrf2 expression which indicated by the downregulation at antioxidant enzymes GSH, CAT levels. though, ROS accumulation due to the decreased activity of CAT and GSH in the MDA-MB-231 cells exposed to radiation (Komorowska et al., 2021; Loenhout et al.,2020; Nuszkiewicz et al., 2020).

Finally, we demonstrated the effect of I3C plus RES with/without IR on EMT genes expression, the data revealed significant downregulation at E-cad levels and upregulation the expression of Vim levels (fig. 3D-F). several literatures proved that the high expression of Nrf2 leads to elevation of E-cad levels and downregulation the expression of Vim, while the knockdown of Nrf2 led to downregulation at E-cad expression and upregulation the expression of Vim (Bocci et al., 2019; Zhou et al., 2016). In summary, I3C plus RES can acts as a potent radiosensitizer, which enhance the responsiveness of MDA-MB-231 breast cancer cells to radiation therapy. The combined treatment of I3C plus RES and radiation reduces cell proliferation, migration, and survival, which suggests that it could be a potential therapeutic strategy for breast cancer. This research provides new insight into the use of I3C aggregated with RES as a radiosensitizer and highlights its potential usefulness in clinical trial.

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