International Research Journal of Oncology

3(3): 13-27, 2020; Article no.IRJO.59207

Synergistic Effect of 5-Fluorouracil Combined with Naringin in MDA-MB-231 Human Breast Cancer Cells

Thangavel Muthusamy[1,2*], L. Roshini Yadav[2], Satish Ramalingam[3], Ilangovan Ramachandran[4] and Prabakaran Nagarajan[5]

[1]Faculty of Allied Health Sciences, Meenakshi Academic of Research and Education, MAHER (Deemed to be a University), West K.K. Nagar, Chennai, 600078, Tamil Nadu, India.
[2]Faculty of Allied Health Sciences, Chettinad Academy of Research and Education (CARE), Kelambakkam, Chennai, 603103, Tamil Nadu, India.
[3]Department of Genetic Engineering, School of Bio-engineering, SRM Institute of Science and Technology, Kattankulathur, Kanchipuram, 603203, Tamil Nadu, India.
[4]Department of Endocrinology, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, 600113, Tamil Nadu, India.
[5]Department of Biological Chemistry and Pharmacology, The Ohio State University, USA.

Authors' contributions

This work was carried out in collaboration among all authors. Author TM designed the work, wrote Introductions, materials and methods, results and discussion of the manuscript and did 60% of the experiments. Author LRY carried out cell culture and assist for western blot and statistical analysis. Author SR helped out for cell migrations assay. Author IR helped for secondary antibodies for the western blot probe. Author PN given valuable suggestions for the manuscript preparation. All authors read and approved the final manuscript.

Article Information

Editor(s):
(1) Dr. Lomas Kumar Tomar, Galway University Hospital, National University of Ireland, Ireland.
Reviewers:
(1) Oseni Oyediran Ganiyu, Bayero University, Nigeria.
(2) Manar Montasser Mohamed Attia, Suez Canal University, Egypt.
Complete Peer review History: http://www.sdiarticle4.com/review-history/59207

Original Research Article

Received 10 May 2020
Accepted 17 July 2020
Published 31 July 2020

ABSTRACT

Antimetabolite established as a successful therapeutics for advanced-stage breast cancers, but has recurrently shown to exhibit major side effects, which restrict drug therapy interventions. The most widely used chemotherapeutic agent is 5-Fluorouracil, however the recent report suggests that its antineoplastic activity is limited due to drug resistance developed by the cancer cells. The resistance results in the decrease in efficacy of 5-FU to improve either invasive disease-free

**Corresponding author: E-mail: evmthangavel@gmail.com;*

survival or overall survival. In order to enhance 5-Fluorouracil mediated cytotoxicity, we have attempted the combination of the natural compound Naringin with 5-Fluorouracil. The combination of 5-Fluorouracil and Naringin inhibited the proliferation of MDA-MB-231 breast cancer cells determined using MTT assay. Importantly, combined treatment showed synergistic enhancement of breast cancer cell cytotoxicity. We next confirmed the cytotoxicity profile of the combination 5-Fluorouracil and Naringin in MDA-MB-231 breast cancer cells with trypan blue-based cell viability assay and determined that the IC_{50} value is 80 µM. When 5-Fluorouuracil and Naringin incubated there is a 7–10-fold increase in cytotoxic effect on breast cancer cells. These results demonstrates that application of naringin as an adjuvant treatment during 5-Fluorouracil administration might enhance the chemotherapeutic efficacy of 5-Fluorouracil.

Keywords: 5-fluorouracil; Naringin; cytotoxicity; MDA-MB-231 breast cancer cells.

1. INTRODUCTION

The most prevalent type of invasive cancer is human breast cancer (BC) and the second common reason of mortality in women [1-3]. BC is most frequently diagnosed malignancy in women of younger age and predominant among tumors causing mortality in this age span [4]. This issue appears as a major public health threat in developing nations where the prevalence of breast carcinoma is promptly increasing. It is estimated that women of younger age group are the highest percentage of the overall breast tumor patient population compared to developed nations [5]. Obesity, age, initial pregnancy at delayed age, late menopause, early menarche, using of postmenopausal hormones, like progesterone and estrogen, and the incidence of an inherited mutation in the BRCA1 or BRCA2 breast genes are main risk factors for the cause of breast carcinoma [6]. Accordingly, novel methods for the treatment of breast cancer are necessary. Plants are the source to provide us new therapeutic candidate compounds. Recognizing novel and less toxic phytochemical, which particularly destroys cells, which are malignant, can direct to the raise of potentially improved therapy for breast cancer patients [1-3]. Tumor biology studies suggest that breast cancer metastasis involves the detachment of tumor cells from the primary tumor, infiltration of surrounding tissues, invasion into blood vessels, and transport to distant sites, and tumor metastasis in distal organs [4,5]. Studies of the mechanism of breast cancer metastasis provide a theoretical basis for the development of drugs that prevent metastasis [4, 5]. The pyrimidine analog 5-Fluorouracil is an antineoplastic antimetabolite medication, which is the first line of anti-cancer chemotherapeutic agents originated and extensively administrated in the treatment of colorectal carcinoma and breast carcinoma [7]. 5-Fluorouracil mechanism is shown to disrupt the nucleotide metabolism by blocking nucleic acid synthesis. 5-FU as a chemotherapeutic agent is an active metabolite of capecitabine utilized highly for the cure of solid cancer such as mammary tumor [8]. The utilization of drugs such as 5-Florouracil is limited by acquiring drug-resistance and lowering the drug systematic efficacy [8]. Several techniques practiced in attempt to overcome this complication such as the combined use of antitumor metabolites [9,10]. Further, it was recently indicated that joint treatment with natural agents improved the competence and inflated the cytotoxicity levels of several anticancer medications. Naringin [Fig. 1] has showed to prevent the progression of mammary tumor cells MDA-MB-435 *in vitro* and Molecular docking also showed that naringin potentially inhibits estrone sulfatase and attenuates the hormonal prompt of breast malignant cells [11]. Research studies have demonstrated that naringin mediates antitumor action against different cancers (e.g., bladder, breast, cervical, colon, liver, and stomach cancers). It has been previously reported that the anticancer property of naringin might involve antiangiogenic effect, cell cycle modulation, or apoptosis. This complete mechanisms [Fig. 2] by which naringin prevents cell growth of tumors (especially Triple Negative Breast Cancer) is yet to be entirely elucidated [12,13]. In this study, using in vitro models, we report the inhibitory effect of naringin on BC cells. Earlier study demonstrated the experiments on estrogen receptor (+) MCF-7 breast cancer cells and estrogen receptor (-) MDA-MB-231 breast malignant cells have dissolved that naringin possesses both estrogenic (at lower dose) and antiestrogenic (at higher dose) behavior mainly during selectively binding with alpha and beta (ERα and ERβ) estrogen receptors. Siegelin *et al*., reported that naringin (0.1 µmol /L) considerably prevented the discharge of growth factor of vascular endothelium from MDA-MB-

231 breast malignant cells and, compacted the incidence of angiogenesis [14]. Pre-treatment with naringin triggered death receptor, mitochondria-regulated apoptosis and, lower survival of cervical SiHa cancer cells at IC_{50} of 750 µM [11]. In 1928, naringin chemical structure was first interpreted by Asahina and Incubuses as shown in [Fig. 1].

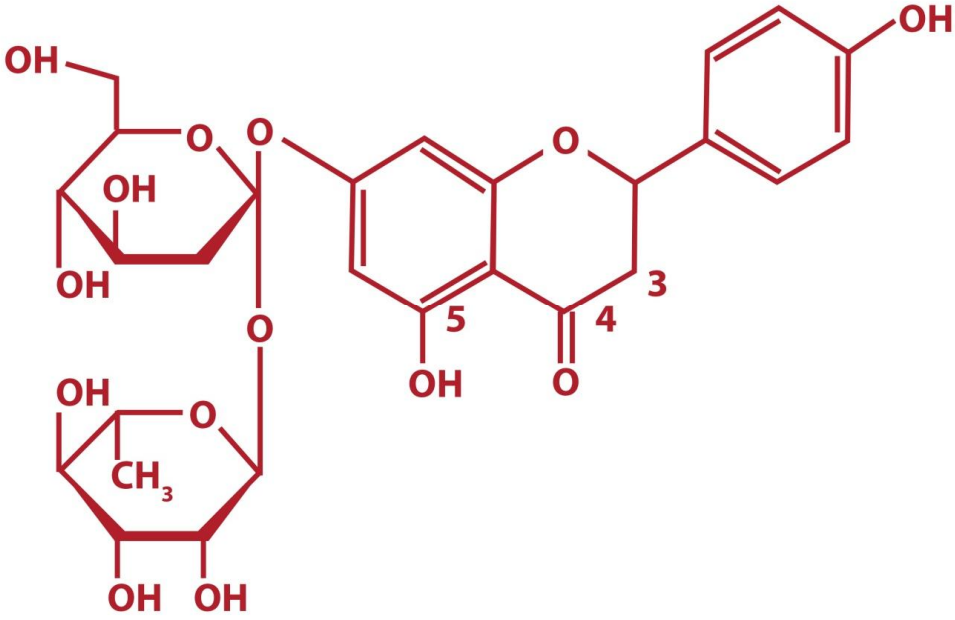


Fig. 1. Structure of Naringin (Flavanone-7-O-glycoside)

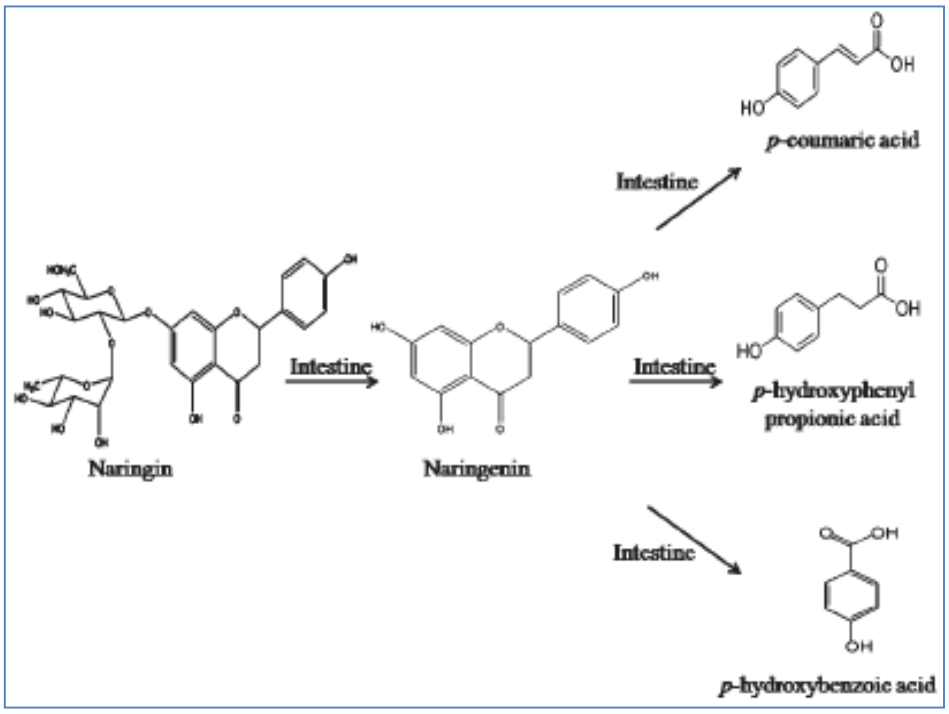


Fig. 2. Anticancer and anti-oxidant activity of naringin

As illustrated earlier naringin inhibit tumor cell growth via promoting cell apoptosis, including triple-negative breast cancer cells, cervical tumor (SiHa) cells and bladder cancer cells. In triple negative breast cancer cells, the pro-apoptotic action of naringin results from G1-stage cell cycle arrest. Suppression of the growth of breast cancer cells by naringin is regulated by inhibition of the β-catenin pathway, leading to a significantly increased p21 level and decreased cell survival [15,16]. Phytotherapeutics involving natural source for therapy of many diseases and also widely used in combination with already existing therapeutics such as for tumors [16]. The combine action or synergistic effects, influence the drug efficacy enhance the anti-cancer drug toxicity for the decreased survival rate of the cancer cells and also drug sensitizing agents with reducing toxic side effects. Although 5-Fluorouracil exhibits as effective anti-cancer chemotherapeutic agent, unique remedial process are required to improve its anticancer effectiveness and alter its cytotoxity, in mixture with additional compounds through the expectation that this synergy strengthen the antiproliferative agitation of 5-Fluorouracil [17]. Synergistic measure of 5-Flurouracil and apigenin on Solid Ehrlich carcinoma (SEC) showed apigenin increases the effect of 5-Fluorouracil on SEC thereby, decreasing drug toxicity and resistance by down-regulating the expression in Myeloid cell leukemia-1 (Mcl-1) important in determining the cell fate by controlling the balance between autophagy and apoptosis, and accompanied 5-Fluorouracil and apigenin exhibits better result than each of 5-Fluorouracil or apigenin unaccompanied [18]. Combination treatment of curcumin with 5-Fluorouracil in adjacent cells not in favor of the cytological effects of 5-FU although it maintains its anti proliferation function towards cancer cells with effects including antioxidant property or its hindrance towards prosurvival directing pathway in breast carcinoma [8]. They showed that curcumin radically hindered 5-Fluorouracil caused cytotoxicity by preserving viability of normal cells. As noted, adding together of curcumin as an pro-treatment to 5-Fluorouracil action in breast malignancy therapy might enhance the chemotherapeutic effectiveness reaction by cooperative administration of high doses of 5-Fluorouracil and lengthy management period and most importantly with DPD deficiencies leading with adverse cytotoxicities that necessitates numerous disruption, or constant execution of curative procedures [8]. 5-Fluorouracil resistance in gastric cancer cells shows that combination of curcumin and 5-Fluorouracil generated synergistic prevention of development and stimulation of effective apoptosis in the challenging cancer cell lines, this demonstrates that the combination of curcumin and 5-Fluorouracil generated synergistic prevention of proliferation and beginning of apoptosis in the resistant malignant cell lines [19].

A natural flavonoid for hepatocellular carcinoma cells in vivo and in vitro signified that oroxylin A raises the sensitivity in hepatocellular carcinoma cells to 5-Fluorouracil [20]. The inhibitory effects of oroxylin A or 5-Fluorouracil on cell viability in HepG2 cells evaluated that oroxylin A and 5-Fluorouracil showed inhibitory effect by a dose-dependent manner [20]. Previous study indicated that in colon cancer SW620 cells melatonin has showed synergistic effect combined with 5-Fluorouracil to restrain the increase of colon tumor by enhancing anti-proliferation, anti-migration, and pro-apoptotic activities [21].

Therefore, in this study, we investigated the positive outcome of 5-fluorouracil in combination with naringin on cellular proliferation and apoptosis using MDA-MB-231 human breast cancer cells.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

5-Fluorourcil was obtained from Sisco Research Laboratories (SRL) (INDIA). MTT-Thiazolyl Blue Tetrazolium Bromide (M5655) (Aldrich Co., USA), Trypan Blue (TC193) and Dimethyl Sulphoxide (DMSO) (TC185) were obtained from Himedia. Breast cancer cells MDA-MB-231 was from National Centre for Cell Science-Pune (NCCS). Dulbecco's Modified Eagles's Medium (AL007), Fetal Bovine Serum, 0.25% trypsin and 0.03% EDTA Ethylenediaminetetraacetic acid (EDTA) was obtained from Himedia. Antibiotic-Antimycotic (100X) (15240096) was obtained from Gibco Life Technologies (USA).

2.2 Cell Culture

Human breast cancer cell line MDA-MB-231 cells were cultured in DMEM medium with FBS of 10% and Antibiotic-Antimycotic of 1% solution incubated at 37^0C with 5% CO_2 until cells reached 75-80% confluent.

Cells were routinely sub-cultured by trypsinization [(0.25% trypsin, 0.03% EDTA (Ethylenediaminetetraacetic acid)], incubated for 5-10 minutes. Upon reaching confluence examined by inverted microscope for bacterial and fungal contamination prior to experiments. Additionally, cells were determined to be free of mycoplasma.

2.3 Cell Proliferation Assay

The MDA-MB-231 cells grown in logarithmic stage were seeded in a 96-well plates at 5×10^4 cells per well and held overnight to adhere, following which a sequence of concentrations of 5-Fluorouracil (20, 40, 80,120,160 in µM) and/or Naringin (40, 80, 120, 160, 200 in µM) and 5-Fluorouracil in combination with Naringin [20+160, 40+160, 80+160, 120+160, 160+160 in µM] was subjected to the well for the incubation period of 48 h. Control experiments were done in MDA-MB-231 cells by addition of DMSO without Naringin. Following 48 h of treatment, cell proliferation was tested with the Thiazolyl Blue Tetrazolium Bromide -MTT assay [22]. In exposure of either 5-Fluorouracil or Naringin and 5-FU in combination with Naringin the cells were incubated again for the next 4h at 37°C. Four hours later it was solubilized by adding DMSO to all the wells also resulted in purple formazan crystals entirely and absorbance values were recorded at 570 nm measured as a percentage of growth in accordance to the control. All the experiments were practiced in triplicate manner. The concentration of the different doses of the drug that lead to 50% inhibition of cell proliferation [IC_{50}] was considered and analyzed for 5-Fluorouracil, Naringin and the various combinations. The IC_{50} value was estimated from the plot.

2.4 Cell Viability Assay

The MDA-MB-231 cells were cultured in 6-well plates at 5×10^4 cells per well and treated with several dose of 5-Fluorouracil in combination with Naringin (20+160, 40+160, 80+160, 120+160, 160+160 in µM) for 48h. Control was reviewed by adding only DMSO to MDA-MB-231 cells. After 48h of treatment period, assessment of cell viability using Trypan blue dye exclusion test allowing for light microscopic quantization of viable cells using hemocytometer. Derived on the principle of exclusion of certain dyes by the membranes of live cells that are intact, suspension of treated cells in PBS contained trypan blue dye to detect the percentage of cells having clear cytoplasm (viable cells) compared to (non-viable cells) with blue cytoplasm. All the experiments were repeated in triplicate. By calculating the viability of cells with CELL VIABILITY (%) percentage formula the % of cell viability of control and 5 -Fluorouracil in combination with Naringin were plotted using graph.

2.5 Cell Migration Assay

The cells were inoculated in six-well plate on reaching 70%-80% confluency; cross lines were made using 100-µL sterile pipette tips and washed three times with sterile PBS to eliminate dislodged cells. Addition of 5-Fluorouracil in combination with Naringin (20+160, 40+160, 80+160, 120+160, in µM) to culture medium. After 0 h, and 48 h, the cultured flask was photographed. Cell migration distance = distance at 0h - distance at 48 h.

2.6 Effect of 5-FU and Naringin Alone or in Combination Effect on the Cell Death Protein Expressions in MDA-MB-231 Cells

Cells on ~90% confluency were seeded on 6-well plates at 5×10^5 cells/plate, after 24h incubation treated with Naringin (160 µM/) and 5-FU (40µM) alone or in combination (an accompanying group of 160µM naringin + 40µM 5-FU) for 48h, as DMEM medium holding equal quantity of DMSO was used as control. For harvesting the culture medium was discarded and using cold PBS buffer the cells were washed twice. Disruptions of pellets of cell made with RIPA buffer and were assembled after centrifugation at 800 ×g at 4 °C for 5 min, and centrifugation of lysates at 9600×g at 4 °C for 15 min for collection of the cytoplasmic and nuclear proteins respectively. Phenylmethylsulfonyl fluoride method was used for determining the protein concentrations.

Protein samples were transferred (40µl each) on PVDF membrane after been loaded on sodium dodecyl sulfate-polyacrylamide gel 12% and separation of proteins by electrophoresis. Blockage of the non-specific binding with 5% milk in TBST buffer for 1 h. Then the membranes were attached with primary antibodies against Caspases-9 (cat# PA5-22252, Thermo Fisher Scientific), PARP (Cat# P-7605, Sigma Aldrich), Bad (Cat# PA5-17896 Sigma Aldrich) Bax (PA5-70418, Sigma Aldrich) or β-actin (Cat# MAB1501 Sigma, Aldrich) [1:1000] overnight at 4°C.With 1× TBST the membrane was washed three

times, which was followed by culturing with secondary antibodies [1:3000 dilution] for 1 h at room temperature and washed again three times in 1× TBST. By means of an ECL system protein bands were visualized. With Quantity One software [Bio-Rad Laboratories, Hercules, CA, USA] the grayscale values of the bands were analyzed.

2.7 Statistical Analysis

All data analyzed with P-values with statistical significance confirmed when $P < 0.05$.This was marked by using Graph Pad Prism [Version 7.0] performing t-tests on triplicate samples.

3. RESULTS AND DISCUSSION

3.1 The Anti-proliferation Studies of 5-FU or Naringin in MDA-MB-231 Human Breast Cancer Cells

The first line drug 5-Fluorouracil is an anti-neoplastic antimetabolite manner of treating in clinical practice. Increasing chemo resistance of MDA-MB-231 cells to available drug therapeutics, including 5-FU. Exploring novel chemotherapeutic regimens to cure advance breast cancer including metastatic breast cancer is of major concern [23]. 5-Fluorouracil has reported to be an efficient tumor drug by playing as an important blocker of nucleic acid construction by controlling thymidylate synthase. Transformation of 5-Fluorouracil to its functional constituent, 5-fluoro-dUMP, opening of dUMP is shut into its nucleotide-binding site of the enzyme and inhibiting dTMP synthesis consequential in unbalancing deoxynucleotide pool preventing DNA synthesis [23]. Even though 5-Fluorouracil is broadly applied in the management of array of breast and colorectal malignancy, the reaction rates are in-sufficient as a first-line administration for advanced cancers. To progress ahead on these consequences, 5-Fluorouracil joint with current drug therapies, such as Oxaliplatin and Irinotecan eminent response of 40–50% [24-25]. Agreeing upon this rationale, combination of 5-Fluorouracil along with phytochemical can secure a cancer drug initiative, restricting adverse side effects and advance progression [26-28]. Which in our accordance by combination with 5-Fluorouracil and naringin the results obtained signify the dosage and time dependent inhibiting process of MDA-MB-231human breast cancer cell proliferation at concentrations higher than 20 µM compared with that produced by treatment with 5-FU alone. Naringin class of flavonoids and particularly to the subgroup of flavanone and as water dissolving molecule, passive transport is found to take part as major role in the biological process [15]. The development of therapeutics against cancer and to elevate the productivity of 5-Fluorouracil, anticipation of its regulation combined with other compounds might improve the growth inhibition action of 5-FU. Naringin is one such product derived from natural source is used, shown to arrest prosurvival signals in breast carcinomas [16]. The effects of 5-FU or naringin on cell growth of MDA-MB-231 human breast cancer cells by Thiazolyl Blue Tetrazolium Bromide assay. Regardless of exposure time a small dose-dependent antiproliferative effect indicating an IC_{50} of 80 µM of 5-FU [Fig. 2A]. Whereas, naringin in a dose and time dependent method [$p<0.05$ Fig. 2B] efficiently inhibited cellular proliferation and cells exposed to 160 µM naringin for 48 hours detected a significant effect. For investigation of conditions required to induce synergistic effects between two compounds, proliferation of cell was determined in the culture exposed to 5-FU at concentrations (20, 40, 80, 120, 160 µM) and naringin at its IC_{50} of 160 µM. 5-Fluorouracil+Naringin (20+160, 40+160, 80+160,120+160,160+160 µM) [Fig. 2C]. Compact 56-70% decline in proliferation with combination treatment, than by 5-FU alone was found.

In vitro experiments of naringin on cancer cell lines such as MCF-7 and MDA-MB-231 exists anti proliferation mechanism [16]. Cell cycle changes, anti-angiogenesis, or stimulation of apoptosis implicated in the tumor inhibition action of naringin, however the exact mechanisms of naringin acting upon cell growth of tumors (such as MDA-MB-231) yet to be completely explained [29]. In current report, we explained the beneficial target of 5-FU and naringin on MDA-MB-231 cells *in vitro* models.

3.2 Effect of 5-FU or Naringin on Cell Growth of MDA-MB-231 a Human Breast Cancer Cells

Diverse range of cellular factors mediates programmed cell apoptosis causing cell death aided with characteristic morphology and cell death [30]. Consistently, Li *et al*. confirmed the anticancer effect of naringin in three (TNBC) cell lines and on tumor model of mouse xenograft. For the involvement in cellular apoptosis, cysteine acid proteases family protein caspases are termed as “executioner” proteins, initiator caspases once activated (like caspases 8 and 9)

slice and operate downstream effector caspases (for example caspases 3 and 7), results in fragmentation of PARP nuclear proteins, and carry cell apoptosis [11]. To additionally explore providing reduced cell proliferation the induction of cell apoptosis. The Analysis of apoptosis using trypan blue for MDA-MB-231 cells [Fig. 3]. Following combined treatment 5-Fluorouracil+Naringin (20+160, 40+160, 80+160, 120+160, 160+160 µM) for 48h, significantly ($p<0.001$) observation of apoptosis arbitrated cell death in breast cancer cell, contributed to the decreased cell feasibility. Enumeration of BT-549, MDA-MB-468 and MDA-MB-231 TNBC cells with various concentrations of naringin, has shown to be significantly inhibited in cell proliferation [31]. To investigate if the reduced cell propagation was because of cell apoptosis initiation, MTT examination of MDA-MB-231 cell apoptosis following action with 5-Fluorouracil and naringin for 48 h, contributes to the reduced cell feasibility. Reports suggesting that naringin exert its anticancer property by blocking the Akt pathway. Similarly, quercetin and genistein flavonoids seem to play a role in Akt signaling, serve in restraining cell proliferation and apoptosis of cancer cells [29].

3.3 5-FU or Naringin Decreases Breast Cancer Cell Migration

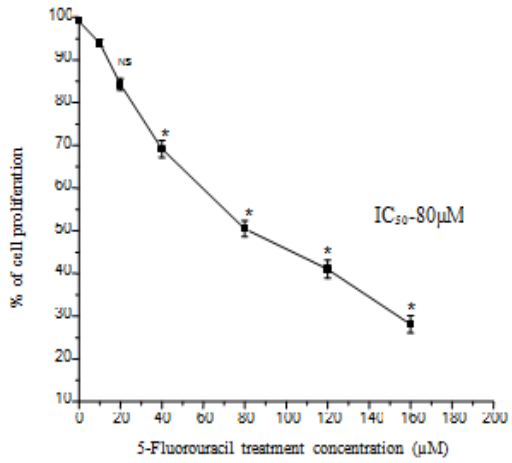
In this study the main purpose is to determine whether 5-Fluorouracil or naringin combinations induce variations in movement of MDA-MB-231 human breast cancer cells. Earlier study results showed cell proliferation experiments, synergistic treatment with 5-FU and apigenin accelerated apoptotic cell death [17]. Similarly our current study, 5-fluorouracil in combination with naringin increased collectively the apoptosis in a naringin dose dependent order. Thus, preventing cell proliferation, cell migration and cell viability may consider as a significant technique. Deriving on the obtained results, the current study is the first to show that MDA-MB-231 human breast cancer cells treatment with 5-FU combined with naringin effected the down-regulation of ErbB2 and Akt signaling [32]. Genetic constituents account to aggressive toxicities of 5-Fluorouracil. As reported that rigorous 5-FU toxicity in 71% of 80 patients shows decreased Dihydropyrimidine dehydrogenase activity, signifying that impaired Dihydropyrimidine dehydrogenase is a chief donor to 5-Fluorouracil correlated unfavorable effects [10]. Antioxidant nature of naringin implicates chemoprotective effects for many organs, accumulation of naringin with 5-FU secured distinctive cells from the cytotoxic side effects of 5-Fluorouracil at the same time as regulating its cell death activity towards tumor cells [33]. We reported here that upon working of various concentrations that (40, 80, 120, 160 µM) of the antimetabolite 5-Fluorouracil, interrupting typical actin organization and inactivity of migrating cells elongated process characteristic of control cells and injury zone trans-location is altered, but not inhibited. 5-Fluorouracil increased concentration (1.0 mM) discernible stress fibers formation was restricted and prevented cellular movement through wound repair the combination groups [Fig. 4 A, B, C & D] took <48 hours to coat a wound scratch, in contrast cells in the control groups took >48 hours to coat a wound scratch. As in given condition, adding up of naringin as an adjuvant therapy to malignant breast cells in the company of 5-Fluorouracil treatment may promote outcomes by cooperative high doses of 5-Fluorouracil and/or longer drug exposure.

Certainly, we determined to facilitate naringin extensively lowered 5-Fluorouracil generated cellular toxicity by sustaining cell activity. Nevertheless, at current these novel result imply that the addition of naringin as an adjuvant treatment during 5-Fluorouracil anti-cancer therapy might promote the drug efficacy of 5-Fluorouracil.

3.4 Effect of 5-FU and Naringin Alone or in Combination on the Expressions of Cell Death Related Proteins [Caspase-9, PARP, Bad (Bcl-2-associated Death Promoter)] in MDA-MB-231 Cells

The consequential machinery of tumor cell death mechanism leads to apoptosis, and the dysregulation of which is important root of tumor progression [34]. Han *et al*., (2018) reported that triggering the activation of caspase - dependent apoptosis in tumor tissues with increase in Bax and Bad expression and decline in the expressional level of Bcl - xl, Bcl-2, and XIAP effective in inducing breast carcinoma cell death [35]. Therefore, we next ought to verify the modulation of Caspases, PARP, Bad and Bax protein level expression induced upon 5-FU and naringin alone or in combination for examination of the potentially linked cell death machinery, to evaluate this we have performed western blotting

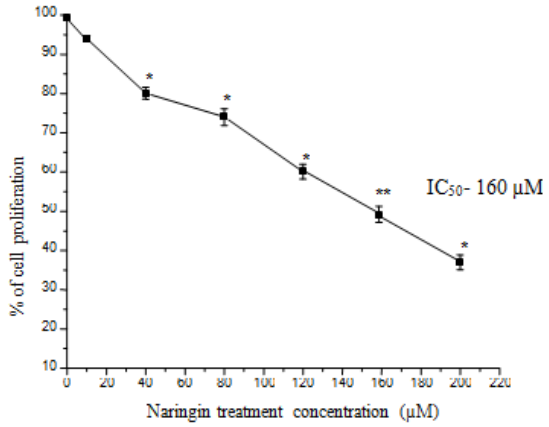
utilizing the MDA-MB-231 cells lysate and the data are reported in Fig. 5. The protein bands intensities of Caspases, PARP, Bad and Bax and β-actin (as control) are summarized in Fig. 5. The increased expression of Caspases, PARP, Bad and Bax proteins was by 44.79%, 47.17%, 45.82% and 60.21% by 5-FU (40 µmol/l), naringin (160 µmol/l), 5-FU (40 µmol/l) + 160 (12.5 µmol/l), respectively. The data revealed that the combination with the same concentrations was more effective to enhance Caspases, PARP, Bad and Bax expression of protein than 5-Fluorouracil and naringin alone ($P < 0.05$). Additionally, the combination of 5-Fluorouracil and naringin presented a concentration-dependent increase in significance in statistical range ($P<0.05$) of the two evaluated concentrations.



A

Fig. 3A. Cells were exposed to 5-Fluorouracil (control, 20, 40, 80,120,160 in µM) and incubated for 48 h .The IC_{50} at 80 µM was determined

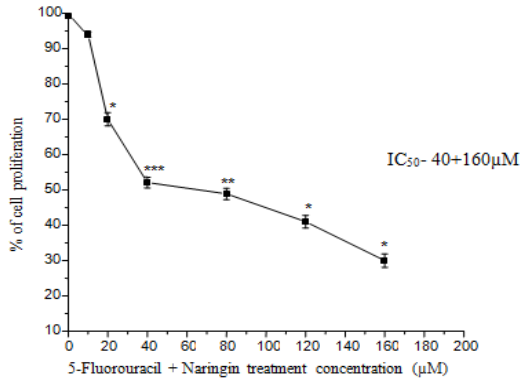
*All data were expressed as (treated vs. control), mean±SD for three replications with NS- no significance, *p<0.05 significantly different from the control group*



B

Fig. 3B. Cells were exposed to Naringin (control, 40, 80,120,160,200 in µM) and incubated for 48 h. The IC_{50} at 160 µM was determined

*All data were expressed as (treated vs. control), mean±SD for three replications with *p<0.05, **p<0.01, significantly different from the control group*



C

Fig. 3C. Cells were exposed to 5-Fluorouracil+Naringin (control, 20+160, 40+170, 80+160, 120+160, 160+160 in µM) and incubated for 48 h. The IC_{50} at 40+170 µM was determined

*All data were expressed as (treated vs. control), mean±SD for three replications with *p<0.05, **p<0.01, ***p<0.001 significantly different from the control group*

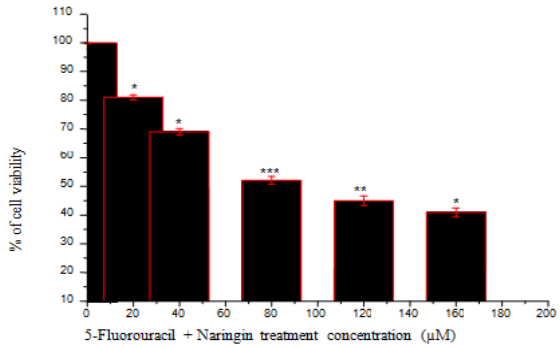
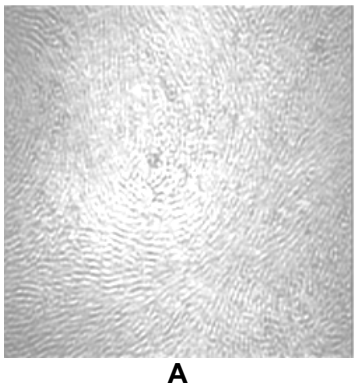


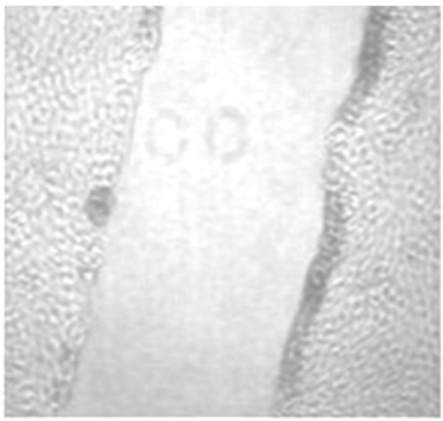
Fig. 4. Cells were exposed to5-Fluorouracil+Naringin (control, 20+160, 40+160, 80+160, 120+160, 160+160 in µM) and incubated for 48 h

*All data were expressed as (treated vs. control), mean±SD for three replications with*p<0.05, **p<0.01, ***p<0.001 significantly different from the control group*



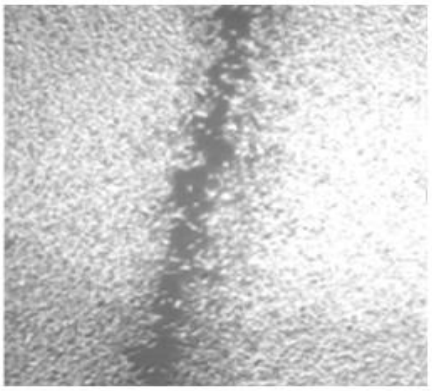
A

Fig. 5A. Effect of 5-FU+Naringin on cell adhesion and migration of human breast cancer MDA-MB-231 cells control - 48 hrs



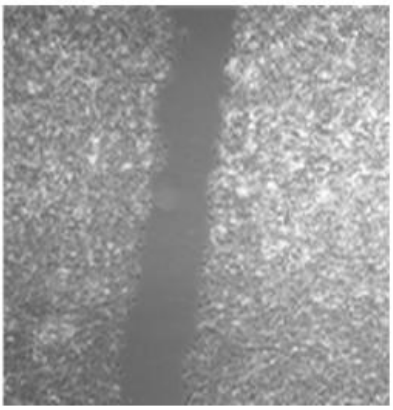
B

Fig. 5B. Effect of 5-FU+Naringin on cell adhesion and migration of human breast cancer MDA-MB-231 cells control - 0 hrs



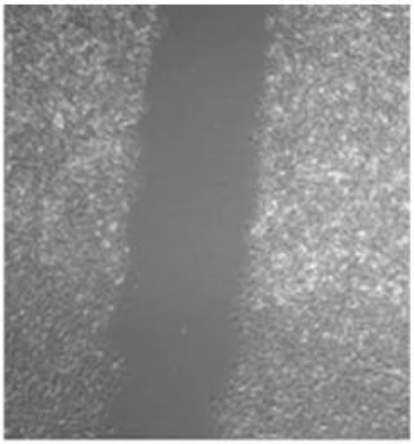
C

Fig. 5C. Effect of 5-FU+Naringin (20+160 µM) 48h on cell adhesion and migration of human breast cancer MDA-MB-231 cells



D

Fig. 5D. Effect of 5-FU+Naringin (40+160 µM) 48h on cell adhesion and migration of human breast cancer MDA-MB-231 cells



E

Fig. 5E. Effect of 5-FU+Naringin (80+160 µM) 48h on cell adhesion and migration of human breast cancer MDA-MB-231 cells

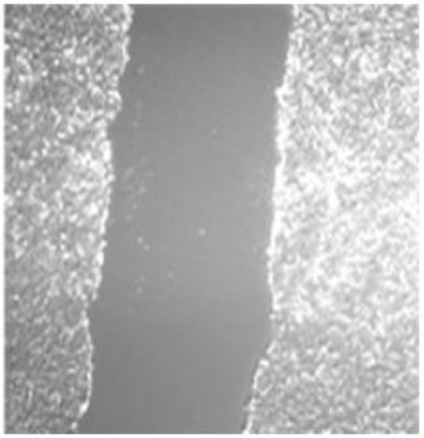


Fig. 5F. Effect of 5-FU+Naringin (120+160 µM) 48h on cell adhesion and migration of human breast cancer MDA-MB-231 cells

Caspase-9 is engaged and rendered by mitochondria-dependent death signal, whereas caspase-2, -8, or -10 activation is induced by death receptors, which turns on caspase-3, -6 and -7 [34]. Then cleaving of PARP into p89 and p24, which hence, leads to fragmentation of DNA and developing apoptosis [36]. Mitochondrial dependent apoptotic pathway with rising expression of apoptotic genes p53, caspase8, caspase-3 and Bax causing apoptosis in MCF7 and MDA-MB-231 cells with mixture of resveratrol and raloxifene [37]. Current results show that 5FU + naringin affected the mitochondrial membrane potential. Western blot analyses indicated that there is an increase in caspase-9 and PARP activity following the combination treatment. Therefore, 5-FU and naringin promoted apoptosis by regulating mitochondrion-mediated pathway.

Disruption of MMP by release of cytochrome c takes place when Bax, a pro apoptotic protein is translocated to the mitochondrial outer membrane. By contrast, in order to prevent the process anti-apoptotic Bcl-2 protecting the mitochondrial integrity. Stimulating Bax level SN38 resulted in apoptosis [38]. The initial Bcl-2 homologue to be recognized Bax inhibits the anti-apoptotic function of the Bcl-2 protein; inducing apoptosis through antagonizing the preserving effects of Bcl-2 [39].

Previous study demonstrated that the Bax to Bcl-2 ratio is a key aspect of facilitating apoptosis

thus, the recognition of the differential expressional level of Bax and Bcl-2 by immunohistochemistry associating the radio sensitizing property of gemcitabine [40]. Usage of gemcitabine could down regulate Bcl-2 protein expression and up regulate Bax protein expression to impact degrees ($P < 0.05$). Their results indicated that major impact on triggering the apoptosis response of tumor cells was through the combination of gemcitabine and radiotherapy rather than the single functioning of either radiotherapy or gemcitabine [40].

Over the last decade previous findings have shown that several regulatory proteins of apoptosis contain other functions distinct to apoptosis [41]. Studies established that cyclin D1 synthesis inhibition imposed G1 phase block via BAD in breast cancer cells which is situated in the nucleus and cytoplasm of normal breast tissue signifying a physiological part for nuclear BAD [41]. Distinguishing the link in BAD/p-BAD declined expression in breast carcinoma and the involvement of enhanced BAD expression with prolonged survival. As breast cancers patients

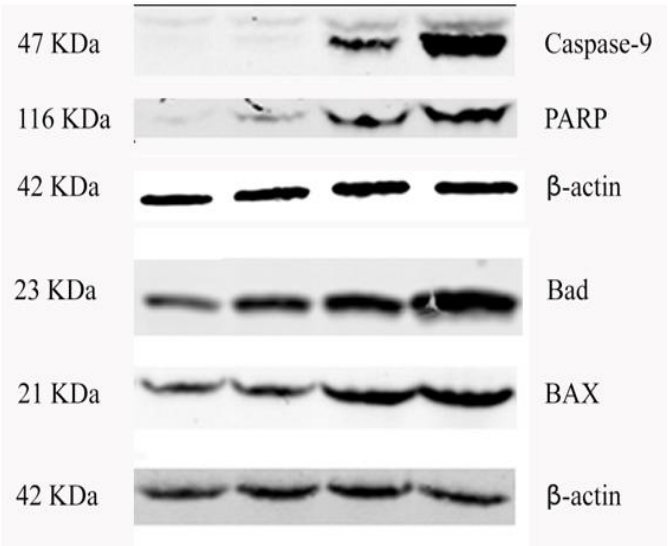


Fig. 6. Western blot analysis for Caspase-9, PARP, Bad, and Bax -induced signaling leads to apoptotic cell death 5-FU combination with naringin induces protein expression

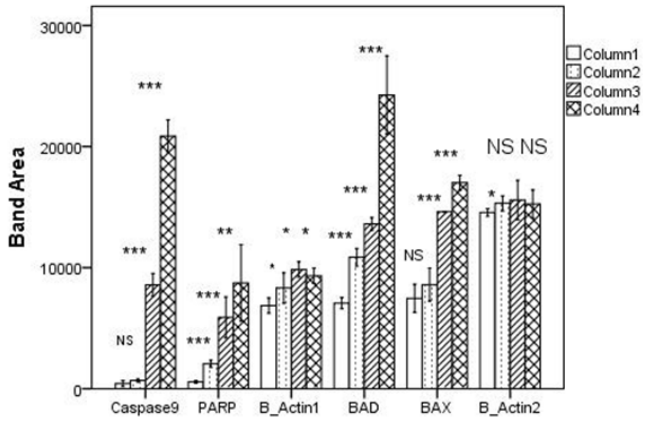


Fig. 7. Western blot quantification analysis for Caspase-9, PARP, Bad, and Bax -induced signaling leads to apoptotic cell death 5-FU combination with naringin induces protein expression

*All data were expressed as (treated vs. control), mean±SD for three replications with *p<0.05, **p<0.01, ***p<0.001 significantly different from the control group. Actin was used as loading control*

comprising high BAD protein level extended survival and which is associated directly to metastasis, it is plausible that lessen metastatic potential could be linked with an improved BAD expression [42]. In addition, Mary *et al*., (2018) have clearly demonstrated that BAD over expression due to induction of mitochondrial-dependent apoptotic pathway inhibit cancer cell survival [43]. The combination therapies appear to have boosted the treatment of cancer. Therefore, by integrating naringin along with low-dose 5-FU could be an effective therapeutic strategy for the treatment of breast cancer is very essential to consider the protected therapeutic window of naringin.

4. CONCLUSION

In conclusion, the current study contributed that low-dose 5-Fluorouracil combined with naringin inhibits proliferation and cell viability more efficiently, compared with 5-FU or naringin alone. Also Naringin potentiates the antiproliferative effect of 5-Flurouracil at a minimal remedial dose, that will decrease the cytological toxicity of 5-FU, providing a valuable novel combination for treating patients with breast cancer.

DISCLAIMER

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and our country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

ACKNOWLEDGEMENT

This work was supported by Chettinad Academy of Research and Education [CARE], Chettinad University, Kelambakkam Chennai-603103, India, who have provided all chemicals and reagents.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rusolo F, Capone F, Pasquale R, Angiolillo A, Colonna G, Castello G, et al. Comparison of the seleno-transcriptome expression between human non-cancerous mammary epithelial cells and two human breast cancer cell lines. Oncol Lett. 2017;13:2411-7.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int. J. Cancer. 2010;127:2893-917.
3. Lu T, Gu M, Zhao Y, Zheng X, Xing C. Autophagy contributes to falcarindiol-induced cell death in breast cancer cells with enhanced endoplasmic reticulum stress. PloS One. 2017;12:e0176348.
4. Lambertini M, Goldrat O, Barragan-Carrillo R, Viglietti G, Demeestere I, Villarreal-Garza C. Viable options for fertility preservation in breast cancer patients: a focus on Latin America. Rev Invest Clin. 2017;69:103-13.
5. Ko BS, Noh WC, Kang SS, Park BW, Kang EY, Paik NS, et al. Changing patterns in the clinical characteristics of Korean breast cancer from 1996-2010 using an online nationwide breast cancer database. J Breast Cancer. 2012;15:393-400.
6. Althuis MD, Brogan DD, Coates RJ, Daling JR, Gammon MD, Malone KE, et al. Breast cancers among very young premenopausal women (United States). CCC. 2003;14:151-60.
7. Guo XF, Yang ZR, Wang J, Lei XF, Lv XG, Dong WG. Synergistic antitumor effect of puerarin combined with 5-fluorouracil on gastric carcinoma. Mol Med Rep. 2015;11: 2562-8.
8. Ferguson JE, Orlando RA. Curcumin reduces cytotoxicity of 5-Fluorouracil treatment in human breast cancer cells. J. Med. Food. 2015;18:497-502.
9. Cameron DA, Gabra H, Leonard RC. Continuous 5-fluorouracil in the treatment of breast cancer. Br J Cancer. 1994;70:120.
10. Curreri AR, Ansfield FJ, McIver FA, Waisman HA, Heidelberger C. Clinical

studies with 5-fluorouracil. Cancer Res. 1958;18:478-84.
11. Li H, Yang B, Huang J, Xiang T, Yin X, Wan J, et al. Naringin inhibits growth potential of human triple-negative breast cancer cells by targeting β-catenin signaling pathway. Toxicol. Lett. 2013; 220:219-28.
12. Kanno SI, Tomizawa A, Hiura T, Osanai Y, Shouji A, Ujibe M, et al. Inhibitory effects of naringenin on tumor growth in human cancer cell lines and sarcoma S-180-implanted mice. Biol. Pharm. Bull. 2005;28:527-30.
13. Kim DI, Lee SJ, Lee SB, Park K, Kim WJ, Moon SK. Requirement for Ras/Raf/ERK pathway in naringin-induced G 1-cell-cycle arrest via p21WAF1 expression. Carcinogenesis. 2008;29:1701-9.
14. Siegelin MD, Reuss DE, Habel A, Herold-Mende C, von Deimling A. The flavonoid kaempferol sensitizes human glioma cells to TRAIL-mediated apoptosis by proteasomal degradation of survivin. Mol Cancer Ther. 2008;7:3566-74.
15. Bharti S, Rani N, Krishnamurthy B, Arya DS. Preclinical evidence for the pharmacological actions of naringin: a review. Planta med. 2014;80:437-51.
16. Chen R, Qi QL, Wang MT, Li QY. Therapeutic potential of naringin: an overview. Pharm. Biol. 2016;54:3203-10.
17. Choi EJ, Kim GH. 5-Fluorouracil combined with apigenin enhances anticancer activity through induction of apoptosis in human breast cancer MDA-MB-453 cells. Oncol. Rep. 2009;22:1533-7.
18. Gaballah HH, Gaber RA, Mohamed DA. Apigenin potentiates the antitumor activity of 5-FU on solid Ehrlich carcinoma: crosstalk between apoptotic and JNK-mediated autophagic cell death platforms. Toxicol. Appl. Pharmacol. 2017;316:27-35.
19. Kang Y, Hu W, Bai E, Zheng H, Liu Z, Wu J, et al. Curcumin sensitizes human gastric cancer cells to 5-fluorouracil through inhibition of the NFκB survival-signaling pathway. Onco Targets Ther. 2016;9: 7373.
20. Zhao L, Chen Z, Wang J, Yang L, Zhao Q, Wang J, et al. Synergistic effect of 5-fluorouracil and the flavanoid oroxylin A on HepG2 human hepatocellular carcinoma and on H 22 transplanted mice. Cancer Chemother. Pharmacol. 2010;65:481.
21. Ogasawara M, Matsubara T, Suzuki H. Screening of natural compounds for inhibitory activity on colon cancer cell migration. Biol Pharm Bull. 2001;24:720-3.
22. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2015; 111:A3-B.
23. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer. 2003; 3:330.
24. Giacchetti S, Perpoint B, Zidani R, Le Bail N, Faggiuolo R, Focan C, et al.Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil–leucovorin as first-line treatment of metastatic colorectal cancer. J Clin Oncol. 2000;18:136.
25. Douillard J, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet. 2000;355:1041-7.
26. Zhong ZF, Tan W, Tian K, Yu H, Qiang WA, Wang YT. Combined effects of furanodiene and doxorubicin on the migration and invasion of MDA-MB-231 breast cancer cells in vitro. Oncol. Rep. 2017;37:2016-24.
27. Aggarwal S, Ichikawa H, Takada Y, Sandur SK, Shishodia S, Aggarwal BB. Curcumin (diferuloylmethane) down-regulates expression of cell proliferation and antiapoptotic and metastatic gene products through suppression of IκBα kinase and Akt activation. Mol Pharmacol. 2006;69:195-206.
28. Du B, Jiang L, Xia Q, Zhong L. Synergistic inhibitory effects of curcumin and 5-fluorouracil on the growth of the human colon cancer cell line HT-29. J CHEMOTHERAPY. 2006;52:23-8.
29. Meiyanto E, Hermawan A, Anindyajati A. Natural products for cancer-targeted therapy: Citrus flavonoids as potent chemopreventive agents. Asian Pac J Cancer Prev. 2012;13:427-36.
30. Cregan SP, Dawson VL, Slack RS. Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene. 2004;23:2785.
31. Ramesh E, Alshatwi AA. Naringin induces death receptor and mitochondria-mediated apoptosis in human cervical cancer (SiHa) cells. Food Chem Toxicol. 2013;51:97-105.

32. Lee WY, Hsu KF, Chiang TA, Chen CJ. Phellinus linteus extract induces autophagy and synergizes with 5-fluorouracil to inhibit breast cancer cell growth. Nutr. Cancer. 2015;67:275-84.
33. Lim SC, Choi JS. Effects of naringin on the pharmacokinetics of intravenous paclitaxel in rats. Biopharm Drug Dispos. 2006;27: 443-7.
34. R Prosperi J, H Goss K. A Wnt-ow of opportunity: Targeting the Wnt/β-catenin pathway in breast cancer. Curr Drug Targets. 2010;11:1074-88.
35. Han B, Jiang P, Liu W, Xu H, Li Y, Li Z, et al.Role of Daucosterol Linoleate on Breast Cancer: Studies on Apoptosis and Metastasis. J Agric Food Chem. 2018;66: 6031-41.
36. Gambi N, Tramontano F, Quesada P. Poly (ADPR) polymerase inhibition and apoptosis induction in cDDP-treated human carcinoma cell lines. Biochem Pharmacol. 2008;75:2356-63.
37. Mirzapur P, Khazaei MR, Moradi MT, Khazaei M. Apoptosis induction in human breast cancer cell lines by synergic effect of raloxifene and resveratrol through increasing proapoptotic genes. Life Sci. 2018;205:45-53.
38. Soldani C, Lazzè MC, Bottone MG, Tognon G, Biggiogera M, Pellicciari CE, et al. Poly (ADP-ribose) polymerase cleavage during apoptosis: when and where? Exp. Cell Res. 2001;269:193-201.
39. Fennell DA, Chacko A, Mutti L. BCL-2 family regulation by the 20S proteasome inhibitor bortezomib. Oncogene. 2008;27: 1189.
40. Shen ZT, Wu XH, Wang L, Li B, Zhu XX. Effects of gemcitabine on radiosensitization, apoptosis, and Bcl-2 and Bax protein expression in human pancreatic cancer xenografts in nude mice. Genet Mol Res. 2015;14:15587-96.
41. Cekanova M, Fernando RI, Siriwardhana N, Sukhthankar M, de la Parra C, Woraratphoka J, et al.BCL-2 family protein, BAD is down-regulated in breast cancer and inhibits cell invasion. Exp. Cell Res. 2015;331:1-0.
42. Al-Bazz YO, Underwood JC, Brown BL, Dobson PR. Prognostic significance of Akt, phospho-Akt and BAD expression in primary breast cancer. Eur. J. Cancer. 2009;45:694-704.
43. Lazer LM, Sadhasivam B, Palaniyandi K, Muthuswamy T, Ramachandran I, Balakrishnan A, et al. Chitosan-based nano-formulation enhances the anticancer efficacy of hesperetin. Int J Biol Macromol. 2018;107:1988-98.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/59207