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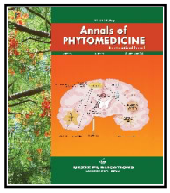

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Elucidation of the molecular mode of action of selected flavonoids (Myricetin and Bergapten) on human breast cancer MDA-MB-231 cells

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Abstract

Myricetin (Myr) and Bergapten (Ber) are two potential anticancer flavonoids. The current study aimed to find out whether the flavonoids Myr and Ber have breast cancer-preventive and therapeutic potential. Myr and Ber separately showed an IC_{50} of 50 µM against MDA-MB-231 (breast cancer) cells while having no discernible impact on normal HEK-293 cells. When compared to untreated cells, Myr and Ber significantly increased the production of free radicals, induced chromatin condensation, early and late apoptosis in MDA-MB-231 cells at respective IC_{50} doses (50 µM). Overall results indicated that Myr and Ber displayed cytotoxicity by reactive oxygen species (ROS) dependent and triggered apoptosis in breast cancer cells. Considering the results of the present study, it is possible to conclude that Myr and Ber possess significant biological activity that would help in increasing the repertoire of effective anticancer drugs with better potency.

1. Introduction

Cancer is a leading cause of death in the globe and is characterized by uncontrolled cell growth and division as well as the dysregulation of signaling pathways, cell invasion that finally results in metastasis. Lung, breast and colorectal cancers are the three most prevalent types of cancer (Bhatt *et al*., 2019; Desai *et al*., 2021). Breast cancer is the second main cause of death globally and the most prevalent cancer in women worldwide (Cao *et al*., 2021). The American Cancer Society has estimated that in 2023, approximately 297,790 new cases will be diagnosed in women in the United States, along with about 55,720 new cases of ductal carcinoma *in situ* (DCIS). Additionally, an estimated 43,700 women will die from breast cancer (Peterson *et al*., 2023). The Cancer Facts and Figure 2023 report includes estimated figure for new cancer cases, deaths, and survivors, as well as information on prevention, early detection and treatment (Kurumety *et al*., 2023). The National Cancer Registry Program of the Indian Council of Medical Research (ICMR) estimates that there are about 22.5 cases of breast cancer per 100,000 women in India. Breast cancer is expected to cause 62,000 deaths in 2020. The stage of cancer determines the available treatment options for breast cancer. Commonly used therapies for breast cancer are hormone therapy, surgery, chemotherapy and targeted drug therapy (Madhav *et al*., 2018). These options are typically combined in most treatment plans. Removing the tumor and some surrounding tissue is involved in surgery, which is the most common treatment for breast cancer. High-energy radiation is used in radiation therapy to destroy cancer cells. To prevent the growth of breast cancer cells, hormone therapy is utilized to hinder the effects of hormones that can promote it (Tewes *et al*., 2021). Drugs are used in chemotherapy to kill cancer cells, while targeted drug therapy focuses on specific proteins or genes that aid in the growth of cancer cells. A treatment option for breast cancer is immunotherapy as well (Naikodi *et al.,* 2021). Treatment options are determined based on various factors, such as the stage of cancer, the patient's age and overall health, and other considerations. However, these treatments can have a wide range of adverse effects, including fatigue, nausea, vomiting, discomfort, changes in appetite and many more (Tewes *et al*., 2021). Cancer resistance is a major challenge in cancer treatment. Resistance to anticancer drugs can result from a variety of factors, such as genetic mutations, epigenetic changes and changes in the tumor microenvironment. Further research and treatment development are necessary due to the high number of drug-resistant in cancers, as cancer cells can develop resistance to traditional therapies (Housman *et al*., 2014). To develop effective treatments, it is crucial to understand the mechanisms that promote or enable drug resistance. Cancer Drug Resistance is a journal that focuses on the pharmacological aspects of drug resistance and its reversal, as well as the molecular mechanisms of drug resistance and the development of new drugs to overcome resistance. Cancer cell proliferation and genetic alterations related to programmed cell death can also lead to multidrug resistance in cancer (Emran *et al*., 2022).

There is ongoing research on the potential of dietary compounds to regulate epigenetic gene expression in cancer and overcome drug

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resistance. Bioactive dietary components have been shown to target epigenetic pathways in cancer, but more research is needed to understand how to optimize their use in cancer treatment (Montgomery and Srinivasan 2019). While some dietary supplements, such as antioxidants, have been associated with increased risk of total mortality and worsened recurrence-free survival in cancer patients, other dietary compounds may have potential in cancer treatment. Complementary and alternative medicine treatments are available to supplement traditional medicine for breast cancer (Bazzan *et al*., 2013). According to some studies, eating plant-based food may help decrease the prevalence of cancer by supplying large amounts of anti-inflammatory ingredients, including phytochemicals and antioxidants (Parmar *et al*., 2010). These substances aid in the body's defense against harmful free radicals that can damage cells and cancer formation (Debnath and Sharma, 2022). Most of the population is treated with medicines or medicinal formulations derived from plants, according to the World Health Organization. In addition, phytochemical extracts with bioactive properties are found in plant extracts, which are commonly used in traditional medicine as safer and more effective alternatives to synthetic drugs (Debnath and Sharma, 2022). They have no side effects, can be used for longer periods of time, and have potential for treating various diseases (Debnath and Sharma, 2022). Hence, screening phytochemicals from various traditional plants is necessary to identify new chemotherapeutic drugs that can precisely, specifically and sensitively target different signaling pathways. Despite the availability of numerous anticancer treatments, their impact on normal and healthy cells causes several side effects, making them difficult and unsafe for the patient.

A class of naturally occurring substances known as flavonoids is abundant in the plant kingdom. They have been explored for their possible health advantages, especially those pertaining to cancer and are well known for their antioxidant properties (Wang *et al*., 2018). Common flavonoids originating from plants include Ber and Myr, which are also found as dietary supplements and are valued for their nutraceutical benefits (Komila *et al*., 2022). Many therapeutic herbs/plants contain these phytochemicals. In essence, bergamot oranges contain the chemical phytocomponent Ber. Myr is present in *Myrica esculenta,* but it is also found in many vegetables, nuts, herbs, and brews like tea, wine, *etc*. (Kabra *et al*., 2019). Although, both compounds have distinctive characteristics, they are frequently utilized to treat a variety of acute and chronic illnesses, such as inflammation, diabetes, cerebral ischemia and atherosclerosis. These compounds are present in many medicinal plants. According to prior studies, Ber has been shown to be useful in treating breast cancer. It has been observed that Ber stimulates metabolic reprogramming of GSK 3 and suppresses cell proliferation in MCF7 cells (Saveria *et al.,* 2020). However, Myr significantly boosts the intrinsic and extrinsic apoptotic pathways in T47D breast cancer cells (Mitra *et al.,* 2020). In accordance with the study, Myr was found to be equally effective as Ber in combating cancer. Thus, the aim of the present study was to investigate the apoptotic activity of flavonoids (Ber and Myr) at effective concentrations in breast cancer cells. The investigational findings might assist in identifying new anticancer drug candidates against breast cancer that could possibly be developed into anticancer agents.

2. Materials and Methods

2.1 *In vitro* biological activity determination

2.1.1 Reagents and chemicals

All the cell culture reagents and chemicals used in the study were of analytical grade as reported in previous studies (Trivedi *et al*., 2018). Myricetin (CAS No. 529-44-2) and bergapten (CAS No. 484-20-8) were purchased from Sigma-Aldrich; doxorubicin hydrochloride (Dox No. 25316-40-9) was also obtained from Sigma-Aldrich.

2.1.2 Maintenance of cell lines

Cell lines (MDA-MB-231 and HEK-293) were purchased from the National Centre for Cell Science (NCCS), Pune and maintained in standard culture conditions by serial passaging in the 25cm^2 culture flask. Cells were in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution, and incubated at 37ºC in a humidified incubator supplied with 5% CO_2.

2.1.3 Sample preparation

Ber, Myr and doxorubicin were dissolved in 0.05% dimethyl sulfoxide (DMSO) and filtered through a 0.22 µm sterile syringe filter stored at 4°C.

2.1.4 Cell culture

For the experiments, cells were trypsinized and seeded at a density of 2×10^6 cells/well for 24 h in 6-well plates (Linbro, MP Biomedicals) for adherence. Cells were exposed to 10-100 µM of Myr and Ber in DMSO for the next 48 h. Suitable untreated controls were also included. All experiments were done in triplicates.

2.1.5 Morphological study

Cells were analyzed and photographed for morphological characteristics under a phase contrast microscope (Nikon Eclipse Ti, Japan).

2.2 Cytotoxicity assay

2.2.1 Cytotoxicity evaluation by trypan blue assay

Live cells were measured through the trypan blue dye. Briefly, 2×10^5 cells/ml were seeded for 48 h. Cells were collected, washed with PBS and mixed with an equal volume (10 µl cells + 10 µl dye) of 0.25% trypan blue dye for 5 min. 10 µl from this solution was used to count the viable cells by using a hemocytometer. The number of live cells per milliliter was calculated using the following formula as reported in previous papers (Trivedi *et al*., 2018):

% Viability = Living cell count/Total cell count × 100

2.2.2 Cell viability detection by MTT assay

MDA-MB-231 and HEK-293 cell lines were seeded at 1.0×10^4 in 96-well plate. In a 96-well plate, cells were treated with both Ber and Myr at doses ranging between 20-100 µM. However, the treatment for standard drug (Dox), the dose range varied from 0.25 µM-1.5 µM. After 48 h of exposer on cells, were cells incubated with 20 µl of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylte trazolium bromide) for 2 h in 96-well plate. Yellow MTT reduced to purple formazan by added to DMSO in plate. Experimental plate absorbance reading were analysed in an ELISA plate reader (Biorad-PW41, USA) at 595 nm.

2.3 Estimation of combination drug on the viability of HEK-293 and MDA-MB-231

CompySyn a computer program based on these methods, is used to automatically determine synergism, additivity, and antagonism of drug combinations (Chou, 2006; Chou, 2010). Drug combinations were depends on the ratio of the IC_{50} values of bergapten and myricetin (50 µM:50 µM = 1:1). Briefly, 1.0×10^4 cells/well were seeded in a 96-well plate and treated with bergapten and myricetin (50 µM:50 µM = 1:1). Ber and Myr in equimolar combinations (5+5, 10+10, 15+15, 20+20, 25+25 30+30, 35+35 and 40 + 40 µM) in separate experiments. Further, MTT assay was performed as explained earlier in section 2.3.2. Three independent experiments with triplicate samples were performed to analyze the drug combination's effect on cancer and normal cells.

2.4 Measurement of intracellular ROS

The intracellular ROS production level was determined using 2,7-dichlorofluorescein diacetate according to the established protocol. Briefly, 2.5×10^4 cells/well were seeded in a 24-well plate and exposed with the bergapten and myricetin compounds (individual/combination) for 24 h. Treated and untreated cells were then incubated with 10 µM DCFDA for 30 min at RT. The cells were visualized under a fluorescence microscope (Zeiss AxioVert 135, US).

2.5 Nuclear condensation assay

Hoechst staining was used to quantify the apoptotic effect of Ber and Myr at their IC_{50} values. Cells were exposed with bergapten and myricetin (individual/combination) manner for 48 h against untreated cells. Then, cells were incubated with Hoechst stain (0.5 µg/ml) for 30 min at 37ºC in the dark and were subsequently analyzed under a fluorescence microscope (Zeiss AxioVert 135, US).

2.6 Evaluation of mechanism of cytotoxicity with AO/EtBr (dual acridine orange/ethidium bromide) assay

MDA-MB-231 and HEK-293 were plated in a 24-well culture plate and treated at their IC_{50} values (ber and myr) for 48 h. Cells were incubated with AO/EtBr (1 µg/ml each) dyes for 10 min at 37°C in a CO_2 incubator and washed with PBS, and visualized under a fluorescence microscope (Zeiss AxioVert 135, US).

2.7 Statistical analysis

All data was provided as mean ± SD. The Graph Pad Prism 5 software was used to calculate statistical significance between distinct groups using a t-test. The data for the *in vitro* analysis were shown as mean ± SD. and statistical significance was determined using a one-way ANOVA.

3. Results

3.1 Effect of Ber, Myr and Dox on morphology and cell viability of MDA-MB-231 cells against HEK-293 cells

MTT assay on MDA-MB-231 demonstrated a significant decrease in cell viability in Ber, Myr and Dox in a dose-dependent manner (Figure 1). Compounds (Ber and Myr) were found their IC_{50} at 50 µM, Dox at 0.50 µM/ml.

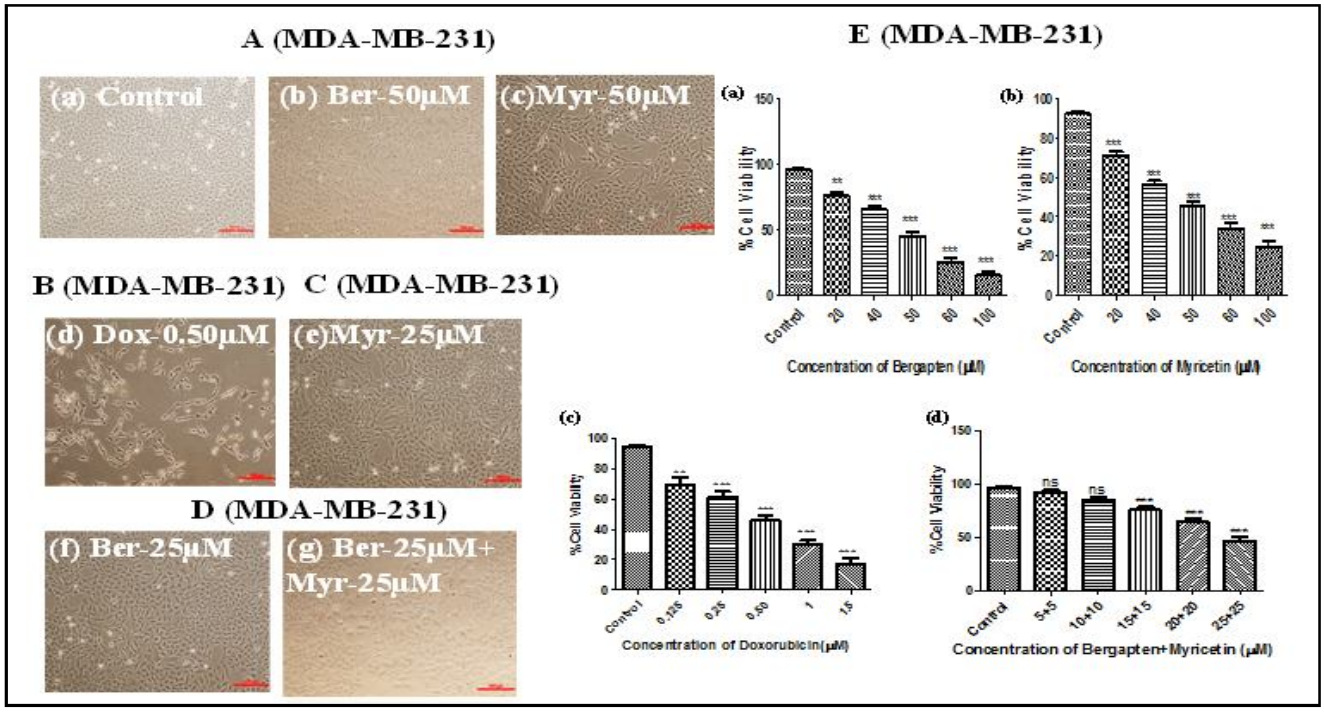

Figure 1: Cytotoxic effects of Ber, Myr and Dox on MDA-MB-231 cells. A. Photomicrographs of untreated (control) MDA-MB-231 cells (a) *versus* those exposed with Ber (b) and Myr (c) for 48 h at their respective IC_{50} values (50 µM). B. Photomicrographs of treated MDA-MB-231 cells with Dox (d) for 48 h at its IC_{50} value (0.50 µM). C. Photomicrographs of treated MDA-MB-231 cells with Myr (e) and Ber (f) for 48 h at 25 µM, respectively D. Photomicrographs of treated MDA-MB-231 cells with an equimolar combination of Ber (25 µM) and Myr (25 µM) for 48 h (g). An inverted phase contrast microscope was used to take the photomicrographs. Scale bar = 100 µm. E. Percent cell viability of Ber (a), Myr (b) and Dox (c) at different concentrations on MDA-MB-231 cells after 48 h incubation. *p* value: ** $p<0.01$ *** $p<0.001$ *versus* untreated control.

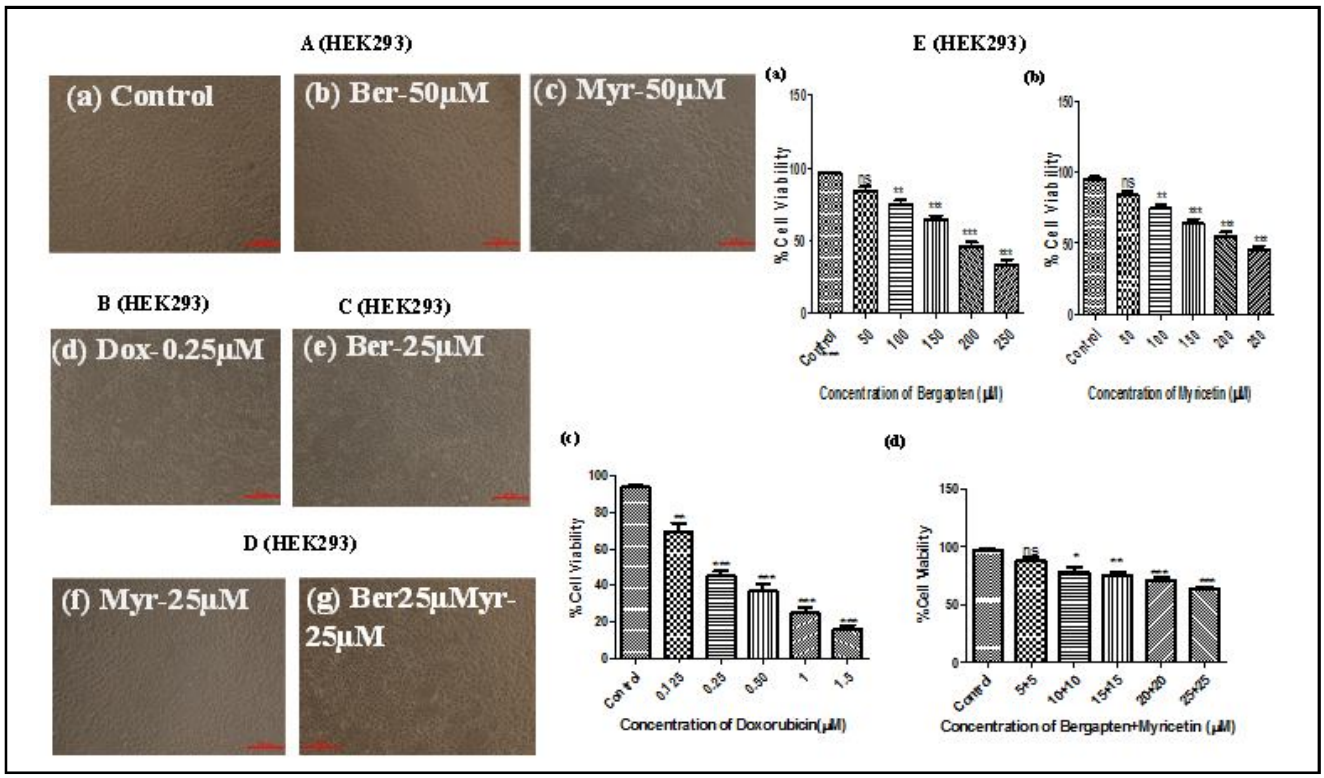

Figure 2: **Effect of Ber, Myr and Dox on normal HEK-293 cells. A. Photomicrographs of untreated (control) HEK-293 cells (a) *versus* those treated with Ber (b) and Myr (c) for 48 h at 50 µM, respectively. B. Photomicrographs of treated with Dox for 48 h at its IC_{50} value (0.25 µM). C. Photomicrographs of cells exposed to an equimolar combination of Ber (25 µM) and Myr (25 µM) for 48 h (e.f). D. Photomicrographs of treated with equimolar combination of Ber (25 µM) and Myr (25 µM) for 48 h. An inverted phase contrast microscope was used to take the photomicrographs. Scale bar = 100 µm. E. Percent cell viability of Ber (a), Myr (b) and Dox (c) Ber (25 µM) and Myr (d) at various concentrations on HEK-293 cells after 48 h incubation *p* value: ** $p<0.01$ *** $p< 0.001$ *versus* untreated control.**

Figure 1 revealed the morphological analysis of untreated versus treated cells with Ber, Myr (50 µM/ml) and Dox (0.50 µM/ml). It is demonstrated from the (Figure 1) that Ber, Myr showed cytotoxic effects on MDA-MB-231 at their IC_{50} values. Treated cells showed morphological variation (round and non-adherent) and normal cells displayed normal morphology with homogenous and equal cell surface at 48 h under inverted microscope. The effects of Myr, Ber and Dox was also evaluated on normal cell (HEK-293) non-significant in the range (10-200 µM) (Figure 2). Interestingly, Dox showed significant toxicity in HEK-293 at 0.25 µM/ml

3.2 Combination dose of Ber and Myr work antagonizing against MDA-MB-231 cell

Ber and Myr treated MDA-MB-231 cells at the range of 5+5, 10+10, 15+15, 25+25, 30+30, 35+35 and 40+40 µM and the IC_{50} of the combination dose of selected compounds was found to be 50 µM (25+25) against MDA-MB-231 (Figure 1D). Another data on normal cell line showed that the IC_{50} of the combination dose of Ber and Myr was found to be 120 µM (64+64) against HEK-293 cells (Figure 2E). Data were found by the using Chou Talalay method for calculating the combinatorial index (CI). Combination data showed an antagonizing effect against MDA-MB-231 cells (Figure 3).

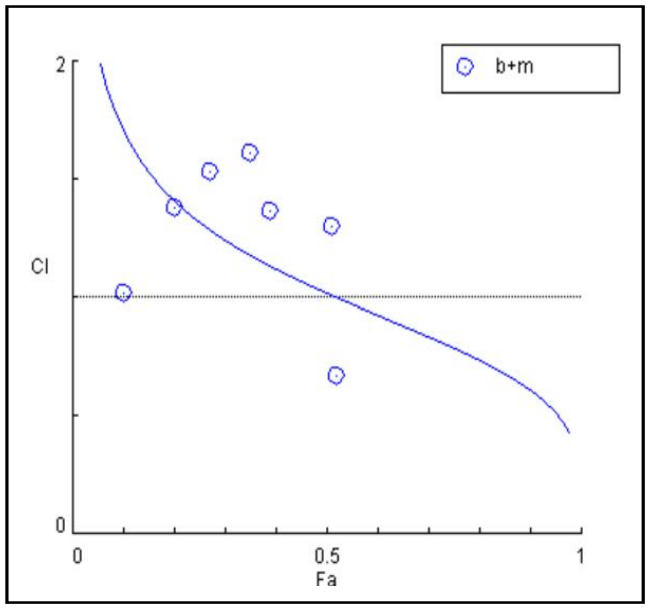

Figure 3: **Combination index pictorial exhibiting antagonist result obtained using CompuSyn software. CI value at IC_{50} (50 µM) of the equimolar combination was calculated as 1.5.**

3.3 Ber and Myr displayed less significant toxicity against HEK-293.

Ber and Myr in an equimolar combination of (25 + 25 µM) showed non-significant cytotoxic effects on HEK-293 cells at the IC_{50} dose of 50 µM.

3.4 Ber and Myr cause oxidative stress in MDA-MB-231.

Ber and Myr showed an increase ROS level at their IC_{50} values (50 µM) by using DCFDA dye, respectively, as compared to untreated control cells (4a and 4b). This data depicted that the growth inhibition observed in the cancer cells, in response to Ber and Myr is might be due to oxidative stress generated by ROS (Figure 4). Quantification of fluorescence intensity of DCFDA staining corresponding to ROS generation was calculated by the using of Image J software (Figure 4a). Scale bar=100 µm (b) Relative fold change in fluorescence intensity (Figure 4b).

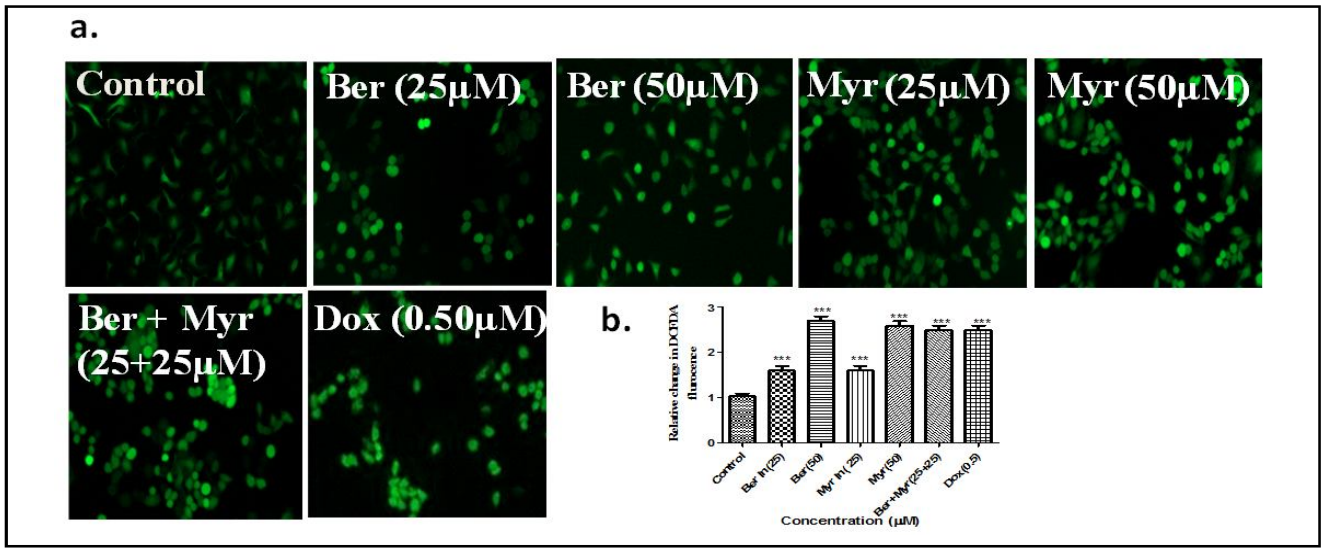

Figure 4: ROS generation in MDA-MB-231 cells exposed by Ber and Myr (a) Photomicrographs depicting intracellular ROS formation in MDA-MB-231 treated cells after 48 h of Ber and Myr treatment at 25 and 50 µM, respectively, Ber and Myr in equimolar combination (25+25 µM) and Dox (0.50 µM). An inverted fluorescence microscope (Zeiss AxioVert 135, US) was used to take the photomicrographs. Scale bar = 100 µm (b) Relative fold change in fluorescence intensity of DCFDHA in treated and untreated MDA-MB-231 cells as measured by Image J software. *** p<0.001 against untreated control.

3.5 Ber and Myr induce chromatin condensation in MDA-MB-231 cells

As evident from Figure 6, effect of Ber and Myr on MDA-MB-231 cells increased chromatin condensation at a dose-dependent manner as compared to the untreated control. Early apoptotic features are chromatin condensation in cell death program which was found in MDA-MB-231 cells at their IC_{50} value of compounds.

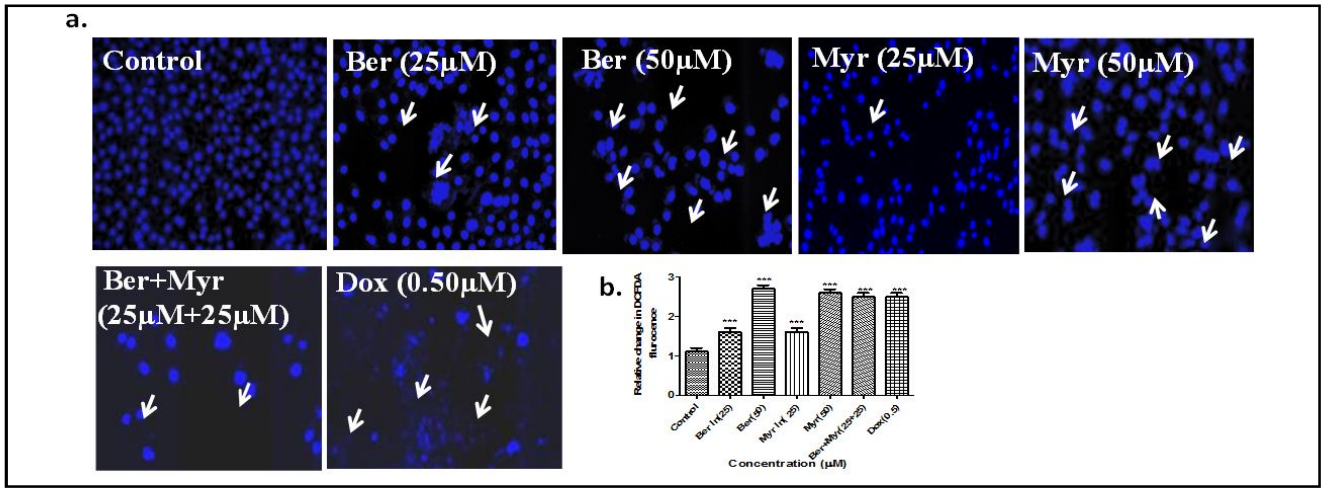

Figure 5: Apoptotic effect induced by Ber and Myr on MDA-MB-231 cells (a) Photomicrographs depicting nuclear chromatin condensation in MDA-MB-231 cells after 48 h of Ber and Myr treatment at 25 and 50 µM, respectively, Ber and Myr in equimolar combination (25+25 µM) and Dox (0.50 µM) (b) Relative fold change in fluorescence intensity of Hoechst 33342 in treated and untreated MDA-MB-231 cells as calculated by Image J software. ***p<0.001 *versus* untreated control.

3.6 Ber and Myr induce early and late apoptosis in MDA-MB-231 cells

Furthermore, Figure 5 showed the effect of compounds on MDA-MB-231 cells at 50 µM. The AO/EtBr double stain showed that control cells had uniformly stained green-colored nuclei indicating that the cells were alive and healthy. Treated cells appeared either green-colored with condensed nuclei indicative of early apoptosis, or orange-red colored cells with condensed nuclei indicative of late apoptosis. MDA-MB-231 displayed early apoptotic (yellow colour cells) features at low doses, whereas late apoptotic (orange colour cells) features were observed at higher doses of the compounds.

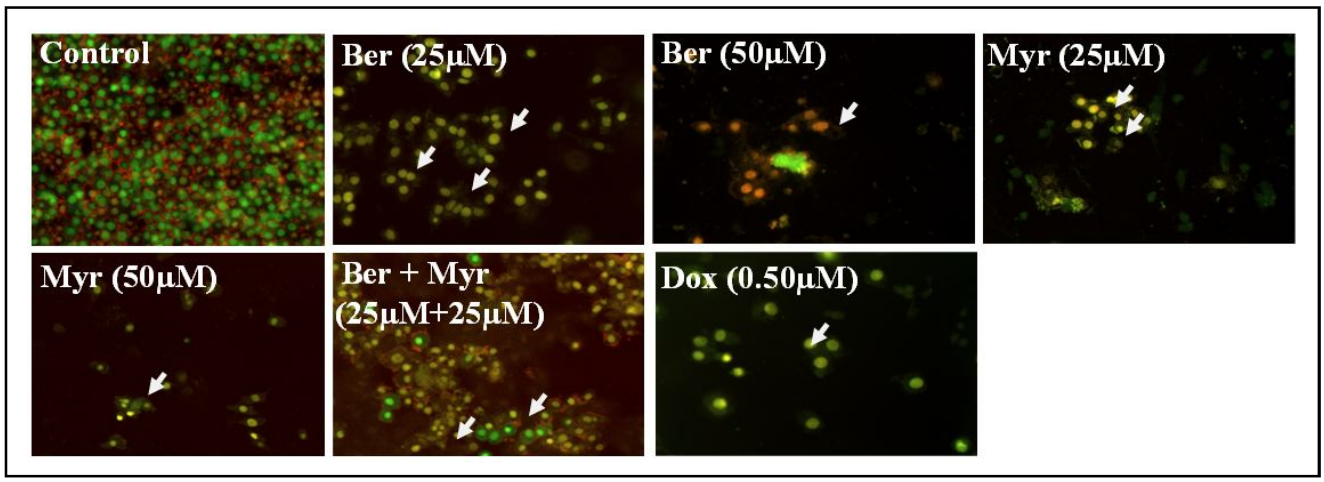

Figure 6: Late and Early apoptosis induction by Ber and Myr on MDA-MB-231 cells. Photomicrographs depicting apoptosis in AO/PI-double-stained MDA-MB-231 cells after 48 h of Ber and Myr treatment at 25 and 50 µM, respectively, Ber and Myr in equimolar combination (25+25 µM) and Dox (0.50 µM). Scale bar = 100 µm.

4. Discussion

The benefits of adopting herbal treatments over synthetic chemotherapeutic medications are numerous, and this is especially true given that synthetic agents severely impair cellular homeostasis and frequently provide therapeutic interventions with low clinical impact (Yi Yin *et al.,* 2013). Therefore, the goal of any therapeutic approach should be to selectively target cancer or tumor cells while causing the fewest number of side effects to normal cells. Due to their safety, potency, and efficacy, secondary metabolites produced from both natural and synthetic sources have recently gained more attention and favor in the field of cancer therapy (Manjinder *et al*., 2014). They also have the potential to be effective anticancer agents. Because it has very few adverse effects, about 80% of the medicine utilized today for various health purposes is derived from plants (Arif *et al*., 2020). Previous studies reporting the cytotoxic activity of Myr and synthesizing its derivatives, termed S4-2-2, were found to possess a cytotoxicity of 78.2 ± 8.1% against the A549 cell line. Ber (5 methoxypsoralen) has anticancer activity (Li *et al*., 2022). Another paper showed that treatment with Ber (50 µM for 48 h) inhibited the cell viability of lung cancer cells by 70%, respectively, compared with the controls. As compared to those results, we also analyzed our cytotoxicity by MTT-assay. We found that Myr and Ber individually have anticancer activity at 50 µM against breast cancer cell lines. Myr (5-30 µM) for 24 h, induces minimal cytotoxicity against normal ovarian cells (Ai Wen *et al*.,2017). Therefore, in the present study, we also analyzed the effect of Myr and Ber on a normal cell line (HEK-231) at 10-100 µM and found that they have non-significant cytotoxicity against the normal cell line. Interestingly, this study also demonstrated that selected flavonoids (Myr and Ber) have anticancer properties against breast cancer cell lines. Furthermore, the morphological variation was analyzed on treated and nontreated normal (HEK-293) and cancer cell lines (MDA-MB-231). Interestingly, myricetin and bergapten were found to be less toxic without significant morphological effects on HEK-293 cells, which depicts that there is minimal toxicity against a normal cell line. Morphological data under inverted phase-contrast microscopy depicted the natural and fibroblastic morphology of untreated MDA-MB-231 cells, whereas Ber and Myr caused a decrease in the number of cells by an alteration in their shape and adherence. These features showed early apoptosis in breast cancer cells. By combining targeted anticancer agents for treatment purposes, new possibilities arise, including overcoming existing resistances due to the increased effectiveness of currently available drugs. So further, we analyzed compounds cytotoxicity by combination methods in MDA-MB-231 cancer cells. Thus, a combination of the two compounds (25+25 µM) was found to have antagonistic activity against MDA-MB-231 cells. Combination results showed antagonictic activity, which did not enhance efficacy compared to the mono-therapy approach in breast cancer cells because MDA-MB-231 is a highly aggressive, invasive, and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks estrogen receptor (ER), progesterone receptor (PR), and BRCA1 expression (Zhaoming *et al*., 2020). Moreover, previous published data by (Sajedi *et al*., 2020) showed that myricetin exerts its apoptotic effects on MCF-7 breast cancer cells by evoking the BRCA1-GADD45 pathway (Nayereh *et al*., 2020). In another study by showed that bergapten induces proteasome-dependent degradation of ER in breast cancer cells through the involvement of SMAD4 in the ubiquitination process (Mauro *et al*., 2012). Moreover, to confirm apoptotic cell death, MDA-MB-231 cells were investigated using DCFDA, Hoechst 33342, and the AO/Etbr assay. One potential reason for the development of cancer is the damage caused by free radicals. Overproduction of ROS can cause macromolecules to oxidize, which is a key factor in DNA mutation, aging, and cell death. The cellular damage associated with cancer may be attributed to increased intracellular ROS generation

(Gabriele *et al*., 2017). Myricetin enhances the apoptosis of triple-negative breast cancer cells, which is mediated by the iron-dependent generation of reactive oxygen species from hydrogen peroxide (Knickle *et al*., 2018). Experiments in the present study for another proof of apoptosis assay involved ROS generation. So, we further analyzed selected compounds (Ber and Myr) that induced ROS as per the dose dependent method in MDA-MB-231. Moreover, nucleus fragmentation was a clear indication of the apoptosis process. Previous papers showed that luteolin, quercetin, and kaempferol induced chromatin condensation and fragmentation of the nucleus in cancer cells (Qiang *et al*., 2015). Myricetin and bergapten have also been shown to induce nuclear condensation in breast cancer cell lines by the Hoechst assay at 50 µM dose. Another study investigated the apoptotic effects of a phytochemical extract from Ajwa dates pulp on MDA-MB-231 cells and found that the extract induced both early and late apoptosis, as quantified by AO/Etbr staining (Khan *et al*., 2021). Further, myricetin and bergapten both promote early and late apoptosis in breast cancer cells at their IC_{50} values, as shown in Figures 6, 7, by AO/Etbr staining. Therefore, our results demonstrated that myricetin and bergapten were cytotoxic to MDA-MB-231. It would be our endeavor in the future to evaluate and assess the anticancer activity of myricetin and bergapten compounds via alternative pathways of apoptosis induction *in vitro* and *in vivo*.

5. Conclusion

These findings may aid in the development of new-targeted chemotherapeutic drugs to achieve successful cancer treatments while also assisting in the prevention of side effects of standard cancer drugs, which are likely to emerge during the course of the study. Compounds may eventually develop into cutting-edge and effective anticancer medications for treating human breast cancer.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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