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EVALUATING THE CYTOTOXIC AND ADDITIVE EFFECTS OF MORIN AND PHYLLANTHIN IN BREAST CANCER CELL LINES: A STEP TOWARDS SYNERGISTIC THERAPEUTICS

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ABSTRACT

Breast cancer is a heterogeneous disease due to which treatment is complicated, as subtypes respond differently to therapies. This study examines the cytotoxic effects of Morin and Phyllanthin in MCF-7 (Luminal-A) and MDA-MB-231 (TNBC) Breast cancer cells. Morin exhibited greater cytotoxicity in MCF-7 cells (IC_{50} = 50.77 ± 1.38 µM) compared to MDA-MB-231 cells (IC_{50} = 59.37 ± 2.22 µM), suggesting increased sensitivity in hormone receptor-positive Breast cancer. Phyllanthin showed similar cytotoxicity in both cell lines (~75.54 µM). The combination index revealed synergy in MCF-7 (CI = 0.673) and an additive effect in MDA-MB-231 (CI = 0.932). Two-way ANOVA analysis shows that Phyllanthin significantly influenced cytotoxicity in MCF-7 (55.1%), while Morin had a greater effect in MDA-MB-231 (48.8%). These findings suggest Morin and Phyllanthin hold therapeutic promise, particularly in ER-positive Breast cancer. Exploring and validating molecular interactions, drug dosage strategies and efficacy of treatments using in vivo models in further studies may provide some better insights into this combination therapy.

KEYWORDS: *Morin, Phyllanthin, Cytotoxicity, Synergy, Combination therapy*

I. INTRODUCTION

Breast cancer is one of the most frequently diagnosed cancers among women globally. With high recurrence and metastasis rates, Breast cancer is posing significant challenges. Despite advancements in therapeutic strategies is Breast cancer second to this is lung and cervical cancer. Breast cancer is the most prevalent cancer globally and presents significant clinical challenges (Table 1). World Health Organization, reports Breast cancer contributes to approximately 24.5% of all cancer cases in women [1]. The occurrence of Breast cancer is increasing steadily. Over 2.3 million new cases are reported annually globally. North America and Europe show the highest incidence rates, but a considerable rise is also seen in low and middle-income countries, likely due to urbanization and changes in lifestyle [2]. Breast cancer remains a major cause of cancer-related mortality, even with early diagnosis and treatment, leading to nearly 685,000 deaths worldwide in 2020 and ranking as the second leading cause of cancer-related deaths [3].

Breast cancer stem cells (BCSCs) are the major contributors to relapse, treatment resistance, and disease progression. BCSCs are a small population of tumor cells with a capacity for self-renewal and resistance

to conventional therapies [3]. Hence, this shows the need for novel therapeutic approaches that can effectively target BCSCs and improve clinical management and therapeutic efficacy.

Table 1: Current Breast Cancer Statistics: Global vs. Indian Perspectives

Category	Global Statistics	Indian Statistics
New Cases (2020)	2.3 million women diagnosed [2]	14.6 lakh new cases projected [4]
Deaths (2020)	685,000 women died [2]	8.09 lakh deaths projected [4]
Leading Cancer Types	Breast cancer: 25% of new cases; Lung cancer: 11% of new cases [2]	Breast cancer: 27% of new cases; Cervical cancer: 18% of new cases [5]
Case Fatality Rate	~50% of cancer patients globally succumb to disease [2]	55% of cancer patients succumb to disease [4]
Key Contributing Factors	Lifestyle factors, tobacco use, and environmental exposures [2]	Lifestyle changes, environmental exposures, tobacco, and alcohol use [6]
Breast cancer Recurrence Risk	The risk of recurrence ranges from 13% to 41% over 5 to 20 years [7]	The risk of recurrence ranges from 15% to 40% over 5 to 20 years, depending on treatment and tumor characteristics [5]
Recurrence and Mortality Rate	25–30% of patients develop recurrence and die due to disease dissemination [7]	20–35% of patients develop recurrence and succumb to disease dissemination [5]

Around 30–40% of Breast cancer patients experience recurrence, especially those with advanced-stage disease, lymph node involvement, or estrogen receptor-negative and HER2-positive subtypes [4]. Especially distant metastasis, recurrence contributes significantly to mortality. Often complications in recurrent cancers are resistant to conventional therapies. Conventional therapies also fail to target Breast cancer stem cells (BCSCs). Given these challenges, novel therapeutic strategies targeting the root causes of Breast cancer, particularly cancer stem cells, are urgently needed. Exploring natural compounds is one promising approach involving Phyllanthin, which may reduce recurrence and improve patient outcomes by disrupting stem cell renewal pathways [10].

Natural bioactive compounds have attracted considerable interest in cancer therapeutics research for their potential to regulate oncogenic pathways while exhibiting minimal to no toxicity. Among these, are Phyllanthin, a lignan derived from *Phyllanthus* species. Morin a flavonoid derived from fruits and vegetables. These phytochemicals have shown to be promising due to their varied pharmacological properties- antioxidant, anti-inflammatory, and anticancer effects [8,9]. By inhibiting tumor cell proliferation, inducing apoptosis, and suppressing critical signaling pathways like *Wnt*/β-catenin, PI3K/Akt, and NF-κB, these two compounds play a crucial role in restricting BCSC survival and metastasis [10,11].

This study evaluates the potential synergism between Phyllanthin and Morin in Breast cancer treatment. By investigating their combined therapeutic impact, this study seeks to determine whether their complementary properties can enhance treatment efficacy, reduce drug resistance, and minimize adverse effects. The study will focus on how the integration of these bioactive compounds may contribute to effective and sustainable strategies in Breast cancer management.

II. MATERIALS AND METHODOLOGY

2.1 Cell Lines Used and Culture Conditions

The human Breast cancer cell lines, MDA-MB-231 (TNBC, metastatic carcinoma) and MCF-7 (Luminal-A, in situ carcinoma), were procured from the American Type Culture Collection (ATCC). These cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cultures were maintained at 37°C in a 5% CO_2 incubator and sub-cultured upon reaching 70–80% confluence.

2.2 Phytochemicals and Drug Formulation

A 2% DMSO stock solution was prepared in culture media. A 2% DMSO stock solution of Morin (Sigma-Aldrich) was prepared, and working solutions were diluted in culture media to the desired

concentrations. A 2% DMSO stock solution of Phyllanthin (Sigma-Aldrich) was prepared, and working solutions were diluted in culture media following the same procedure as Morin. A 10 mM stock solution of Doxorubicin (Sigma-Aldrich) was prepared in DMSO, and working solutions were diluted in culture media to the required concentrations for treatment. A negative control using 2% DMSO was included to confirm that any observed cytotoxic effects were due to the phytochemicals rather than the solvent.

2.3 Cytotoxicity Assays

Cell viability assay was performed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay post-phytochemical treatment. MDA-MB-231 and MCF-7 cells were plated in 96-well plates at a density of 1×10^4 cells per well and allowed to attach for 24 hours before treatment. The cells were then treated with different concentrations of Morin, Phyllanthin, and their combinations for 24 hours. Doxorubicin was used as a positive control, while 2% DMSO served as a negative control.

After the 24-hour treatment, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 hours. The resulting formazan crystals were dissolved in 100 µL of isopropanol containing 0.04 N HCl. Absorbance was measured at 570 nm in a plate reader (BioTek Instruments, USA). IC_{50} were calculated from the obtained values using "Very Simple IC50 Tool Kit (Python version)" software [27].

2.4 Combination Treatment

For combination treatment, phytochemical concentrations were selected based on their respective IC_{50} values, with concentrations below the IC_{50} used for dual combinations. The concentration of one compound was fixed, while the concentration of the second compound was gradually increased. The interaction between the compounds was evaluated as a combination index (CI), calculated by the following formula:

$CI = IC_{50}(\text{drug1combination})/IC_{50}(\text{drug1alone}) + IC_{50}(\text{drug2combination})/IC_{50}(\text{drug2alone})$

Based on the CI values obtained, interactions are classified as synergistic if CI is < 0.8, additive if CI is between 0.8 and 1.2, and antagonistic if CI is > 1.2.

2.5 Statistical Analysis

Experiments were performed in triplicates and the results are expressed as the mean ± standard deviation (SD). IC_{50} values are determined from dose-response curves using the Very Simple IC50 Tool Kit (Python version). Data were analyzed using two-way ANOVA and Dunnett's multiple comparisons test GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA) [27], [28].

III. RESULTS

Table 2: IC_{50} Values Of Morin And Phyllanthin In Breast Cancer Cell Lines
(IC_{50} values are presented as mean ± standard deviation)

Compound	MDA-MB-231 (µM)	MCF-7 (µM)
Doxorubicin	3.2005 ± 5.622 (Figure-1)	1.90681 +/- 1.52(Figure-8)
Phyllanthin	75.54 ± 3.74 (figure-2)	75.54 ± 1.74 (Figure-9)
Morin	59.37 ± 2.22 (Figure-3)	50.77 ± 1.38 (Figure-10)

Table 3: Combination Index (CI) Values of Morin And Phyllanthin In Breast cancer Cell Lines

Compound Combination	MDA-MB-231	MCF-7
Morin + Phyllanthin	0.932	0.673
Effect	Additive	Synergistic

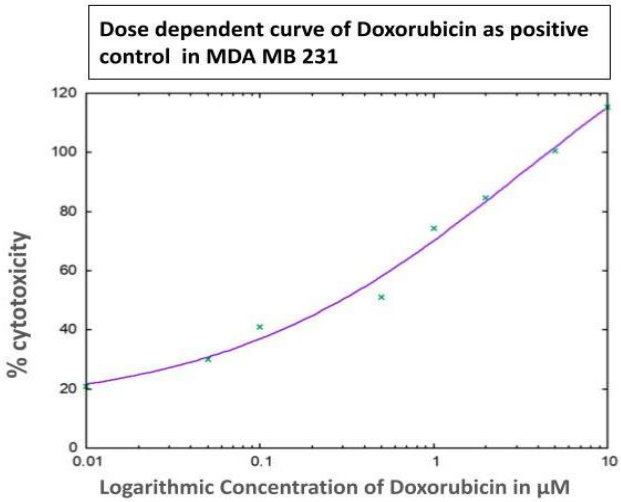

Figure-1: Dose response curve of Doxorubicin as a positive control in MDA-MB-231 cells

The cytotoxic effect of Doxorubicin in MDA-MB-231 Breast cancer cells was estimated using an MTT assay. The X-axis represents the logarithmic concentration of Doxorubicin (µM) and the Y-axis indicates the percentage of cytotoxicity as shown in figure 1. Data points (blue crosses) represent mean values, and the fitted dose-response curve (purple) illustrates the concentration-dependent increase in cytotoxicity. The IC50 value of Doxorubicin was determined to be 3.2005 ± 5.622 µM, confirming its role as a positive control for cytotoxicity in this cell line.

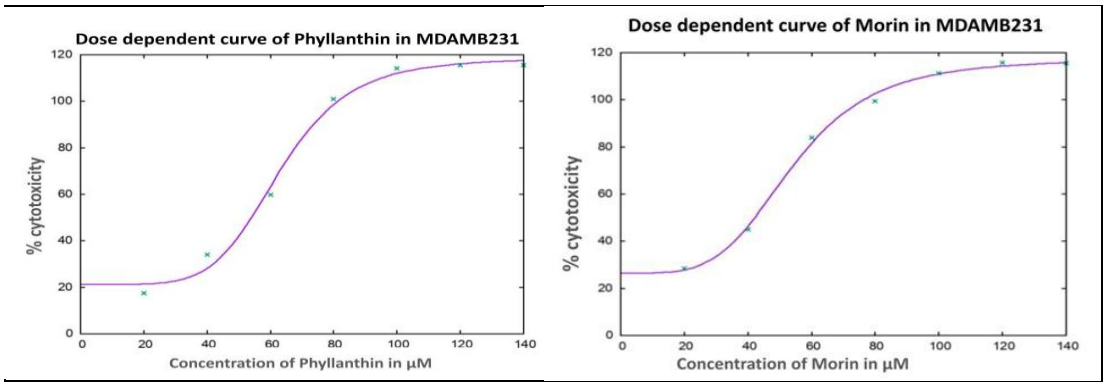

Figure-2: Dose Responses Curve Of Phyllanthin And Morin For Cytotoxicity In MDA-MB-231 Cells

The cytotoxic effect of Phyllanthin and Morin on MDA-MB-231 Breast cancer cells was estimated using an MTT assay. The X-axis represents the concentration of Phyllanthin and Morin (µM) respectively, and the Y-axis is % cytotoxicity as shown in figure 2. Data points (blue crosses) represent mean values, and the fitted dose-response curve (purple) illustrates the concentration-dependent cytotoxicity. The IC50 value of Phyllanthin was determined to be 70.7857 ± 4.915 µM and IC50 value of Morin was determined to be 59.3656 +/- 2.221 µM indicating moderate cytotoxic activity in this cell line.

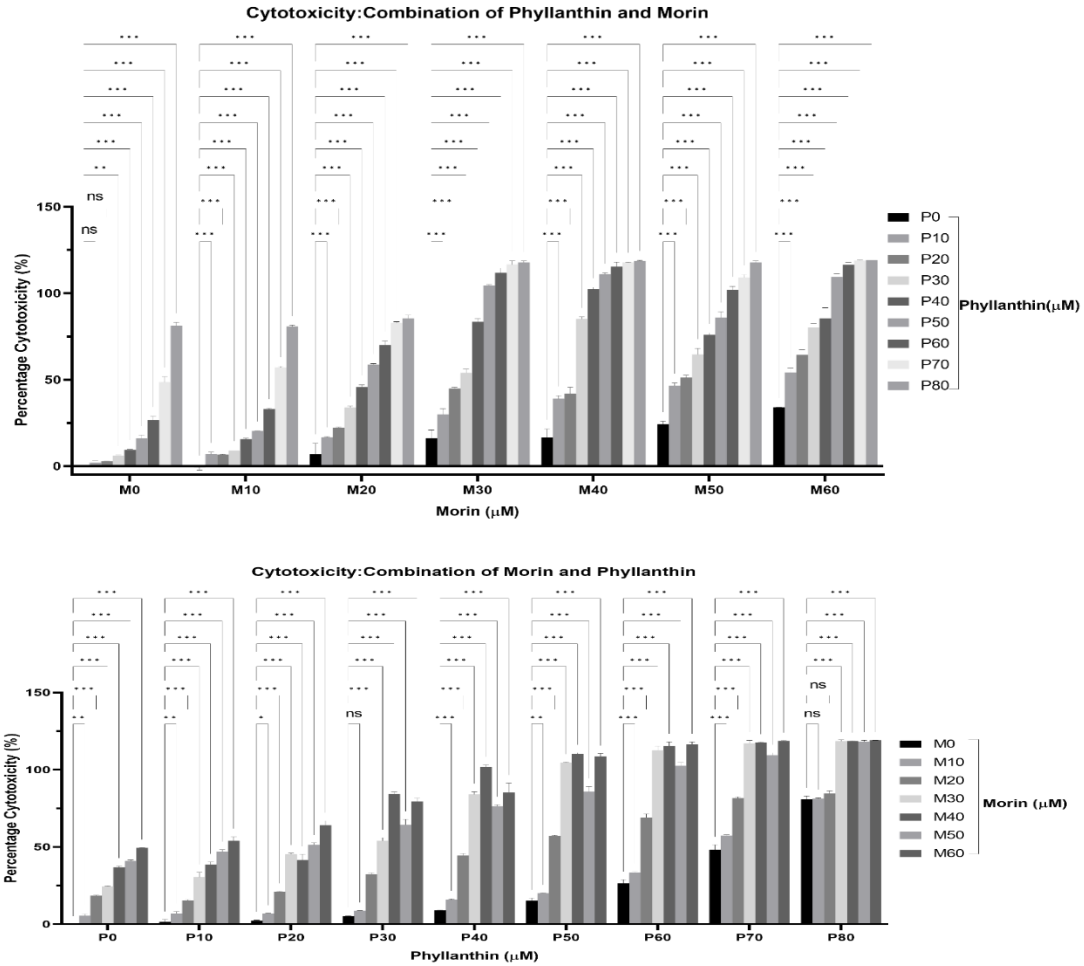

Figure-3: Cytotoxic Effects of Phyllanthin and Morin Combinations on MDA-MB-231 Cells

Cytotoxicity analysis of various combinations of Phyllanthin and Morin on MDA-MB-231 cells at different concentrations (Morin: 0, 10, 20, 30, 40, 50 and 60 μM; Phyllanthin: 0, 10, 20, 30, 40, 50, 60, 70 and 80 μM) as shown in figure 3. The percentage of cytotoxicity was measured and compared for each combination. Statistical significance between groups are indicated by statistical symbols (*p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant). Data points represent the mean ± standard deviation of three independent experiments.

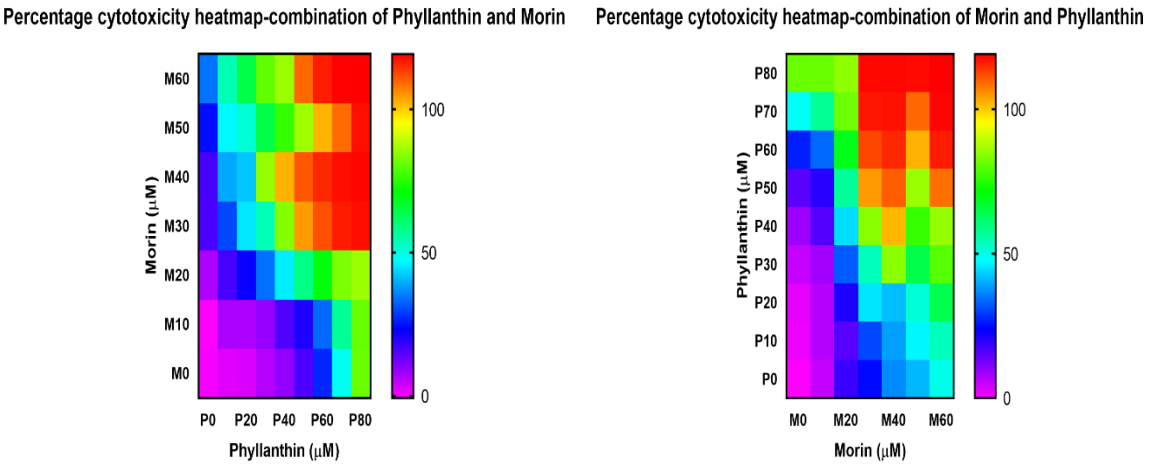

Figure-4: Heatmap Depicting the Cytotoxicity Induced By Combinations Of Phyllanthin And Morin In MDA-MB-231 Cells.

Heatmap depicting the percentage cytotoxicity in MDA-MB-231 cells treated with various concentrations of Phyllanthin (P0, P10, P20, P30, P40, P50, P60, P70 and P80 µM) and Morin (M0, M10, M20, M30, M40, M50 and M60 µM) as shown in figure 4. Cytotoxicity levels are from low - purple to high - red, as indicated by the color scale on the right. Data represent the result of mean values obtained from three independent experiments.

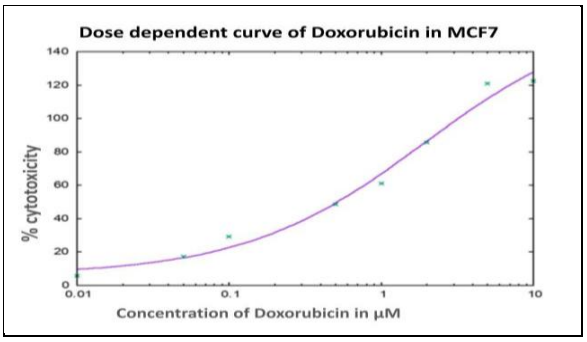

Figure-5: Dose response curve of Doxorubicin as a positive control in MCF-7 cells

The cytotoxic effect of Doxorubicin in MCF-7 Breast cancer cells was estimated using an MTT assay. X-axis represents the logarithmic concentration of Doxorubicin (µM), Y-axis indicates the percentage of cytotoxicity as shown in figure 5. Data points (blue crosses) represent mean values, and the fitted dose-response curve (purple) illustrates the concentration-dependent increase in cytotoxicity. The IC50 value of Doxorubicin was determined to be 1.90681 +/- 1.52 µM, confirming its role as a positive control for cytotoxicity in this cell line.

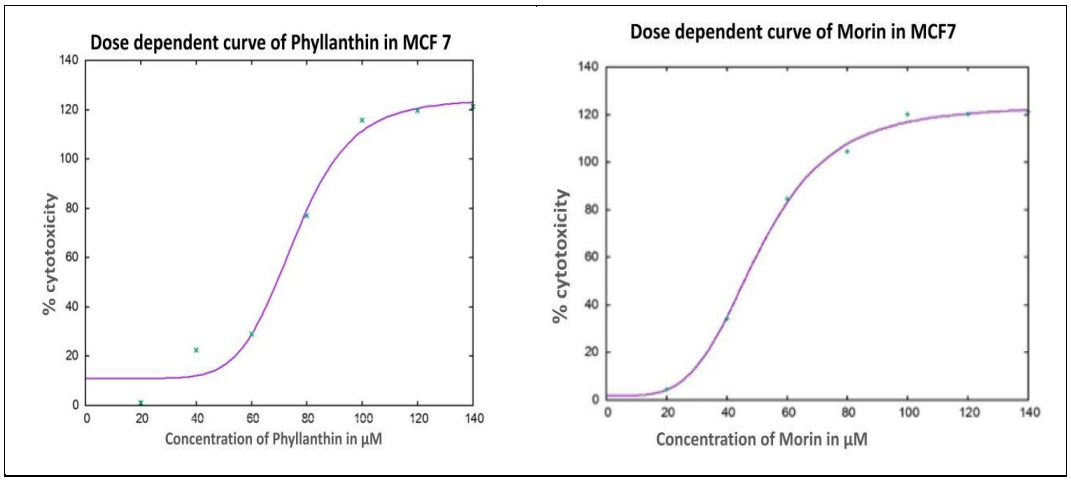

Figure-6: Dose-Response Curve of Phyllanthin and Morin for Cytotoxicity in MCF-7 Cells

The cytotoxic effect of Phyllanthin and Morin on MCF-7 Breast cancer cells was estimated using an MTT assay. X-axis represents the concentration of Phyllanthin and Morin (µM) respectively, Y-axis is % cytotoxicity as shown in figure 6. Data points (blue crosses) represent mean values, and the fitted dose-response curve (purple) illustrates the concentration-dependent cytotoxicity. The IC50 value of Phyllanthin was determined to be 75.5435 +/- 3.738 and IC50 value of Morin was determined to be 50.7726 +/- 1.384 µM indicating moderate cytotoxic activity in this cell line.

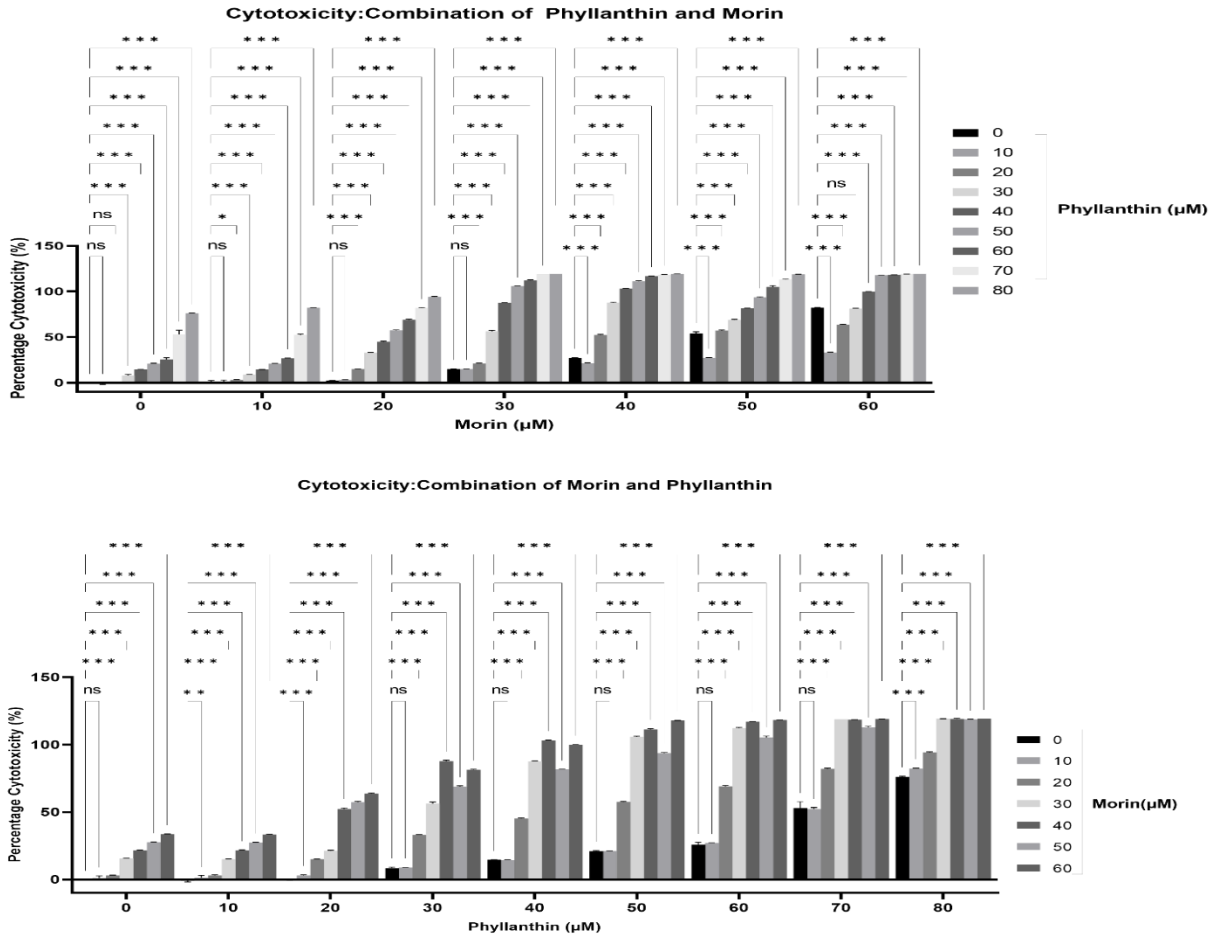

Figure-7: Cytotoxic effects of Phyllanthin and Morin combinations on MCF-7 cells

Cytotoxicity analysis of various combinations of Phyllanthin and Morin on MCF-7 cells at different concentrations (Morin: 0, 10, 20, 30, 40, 50 and 60 µM; Phyllanthin: 0, 10, 20, 30, 40, 50, 60, 70 and 80 µM) as shown in figure 7. The percentage of cytotoxicity was measured and compared for each combination. Statistical significance between groups are indicated by statistical symbols (*p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant). Data points represent the mean ± standard deviation of three independent experiments.

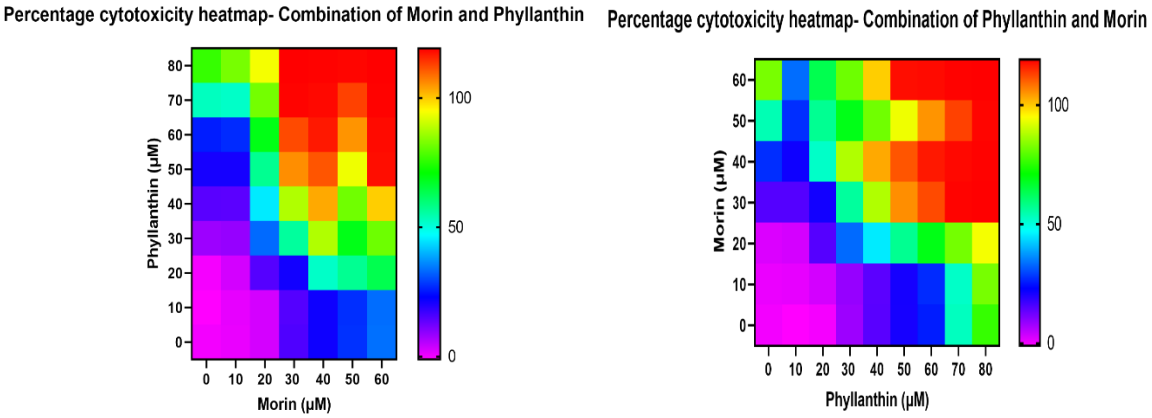

Figure-8: Heatmap Depicting the Cytotoxicity Induced By Combinations Of Phyllanthin And Morin In MCF-7 cells

Heatmap depicting the percentage cytotoxicity in MCF-7 cells treated with various concentrations of Phyllanthin (P0, P10, P20, P30, P40, P50, P60, P70 and P80 μM) and Morin (M0, M10, M20, M30, M40, M50 and M60 μM) as shown in figure 8. Cytotoxicity levels are from low-purple to high-red, as indicated by the color scale on the right. Data represent the result of mean values obtained from three independent experiments.

The half-maximal inhibitory concentration (IC_{50}) values for Morin and Phyllanthin in MDA-MB-231 and MCF-7 Breast cancer cells are presented in Table 2. Morin exhibited a lower IC_{50} in MCF-7 cells (50.77 ± 1.38 μM) compared to MDA-MB-231 cells (59.37 ± 2.22 μM) (Table 2), indicating greater sensitivity of MCF-7 cells to Morin treatment. In contrast, Phyllanthin showed comparable IC_{50} values in both cell lines (75.54 ± 3.74 μM in MDA-MB-231 and 75.54 ± 1.74 μM in MCF-7) (Table 2), suggesting a consistent cytotoxic effect across these Breast cancer subtypes [12].

To estimate the interaction between Morin and Phyllanthin, a combination index (CI) was calculated (Table 3). The results revealed a CI of 0.673 in MCF-7 cells, signifying synergistic effects, whereas a CI of 0.932 in MDA-MB-231 cells indicated an additive interaction. The synergistic effect in MCF-7 cells suggests an enhanced cytotoxic response, potentially mediated through estrogen receptor (ER) signaling [13]. The additive effect in MDA-MB-231 cells implies while the combination retains cytotoxic activity, further dose optimizations or additional agents may be needed to enhance efficacy [14].

The cytotoxic effects of Morin and Phyllanthin were analyzed using two-way ANOVA in MCF-7 and MDA-MB-231 cells. In MCF-7 cells, Phyllanthin contributed to 55.1% of the observed cytotoxicity, while Morin accounted for 37.7%, with a statistically significant interaction effect of 7.2% ($p < 0.001$) (Figure 8). Similarly, in MDA-MB-231 (Figure 4), Morin contributed 48.8% of the variation, Phyllanthin accounted for 45.1%, and their interaction effect was 5.97% ($p < 0.001$) [15].

The most effective combinations were identified based on their observed cytotoxicity rather than IC_{50} values, all of them showing significant reductions in cell viability ($p < 0.001$). Specific concentration ratios of P60-P80 + M40-M60 in MDA-MB-231 (Figure 5) and P60-P80 + M40-M60 in MCF-7 (Figure 9) demonstrated enhanced cytotoxic potential, warranting further mechanistic studies [16].

IV. DISCUSSION

The cytotoxicity of Morin and Phyllanthin aligns with reported studies demonstrating their anticancer properties. Morin is known for its pro-apoptotic and anti-proliferative effects, often mediated through oxidative stress modulation and inhibition of survival pathways [17]. Morin shows higher sensitivity in MCF-7 cells suggesting that its activity may be influenced by estrogen receptor (ER) signaling, suggesting its higher efficacy in ER-positive Breast cancer [18].

Phyllanthin has been reported in previous studies to exhibit anticancer effects through apoptosis induction and inhibition of proliferative signaling [19]. It is more cytotoxic in MCF-7 cells, suggesting that its mode of action may be through cellular metabolism, leading to a differential response between estrogen receptor-positive and triple-negative Breast cancer [20]. Hence dose optimization and additives may improve its metabolism and its therapeutic efficacy.

The synergistic effect (CI = 0.673), observed in MCF-7 cells, indicates that the combination of Morin-Phyllanthin enhances cytotoxicity beyond their individual effects suggesting the mode of acting on Phyllanthin through cellular metabolism which may have been improved by the Morin effect. This may also be attributed to complementary mechanisms, such as oxidative stress induction, DNA damage, or inhibition of oncogenic pathways [21]. In contrast, the additive effect (CI = 0.932) in MDA-MB-231 cells suggests that while retaining its cytotoxicity, the combination may not exhibit strong synergy due to resistance mechanisms mediated in triple-negative Breast cancer (TNBC) [22]. Future studies focusing on molecular interactions and pathway inhibition may provide deeper insights into optimizing this combination for enhanced efficacy in TNBC.

V. CONCLUSION

This study establishes that Morin and Phyllanthin exhibit significant cytotoxicity on Breast cancer cell lines, with MCF-7 cells showing comparatively higher sensitivity. Morin showed a lower IC_{50} in MCF-7 cells, while Phyllanthin had a lesser cytotoxic effect across both cell lines. Combination index (CI) analysis revealed a synergistic effect in MCF-7 cells, suggesting that Morin and Phyllanthin enhance each other's cytotoxic activity, whereas an additive effect in MDA-MB-231 cells was observed.

The findings of this study indicate that combining Morin and Phyllanthin is a promising strategy in Breast cancer therapy, particularly in ER-positive Breast cancer, where synergy enhances therapeutic efficacy. Further studies should explore dose optimization, mechanistic insights, and further additives or carriers such as nanoparticles to determine the clinical relevance of these findings.

VI. SCOPE FOR FURTHER RESEARCH

Focusing on identifying the molecular pathways in the synergistic interaction of Morin-Phyllanthin, particularly in ER-positive Breast cancer. Studies on oxidative stress markers, apoptotic proteins, and signaling pathways could provide deeper insights into their combined anticancer mechanisms [23].

Assessing tumor growth inhibition, bioavailability, and pharmacokinetics using animal models will be crucial for therapeutic validation [24].

The dose-response relationship of Morin and Phyllanthin should be explored further by increased treatment time to optimize their therapeutic window. Evaluating their effects in combination evaluating its effect with standard chemotherapeutic agents may open new avenues in treatment strategies for Triple-negative Breast cancers [25]. Since MDA-MB-231 cells exhibited an additive effect rather than synergy, combining Morin and Phyllanthin with other synergistic phytochemicals or targeted therapies can be explored in future studies [26].

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