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Combinatorial effect of diclofenac with piperine and *D*−limonene on inducing apoptosis and cell cycle arrest of breast cancer cells

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ABSTRACT

Objective: To investigate the potential synergistic activity of diclofenac with piperine and *D*-limonene in inducing apoptosis and cell cycle arrest in breast cancer MCF-7 cells.

Methods: Molecular docking study was conducted to evaluate the binding affinity of diclofenac with piperine and *D*-limonene against p53, Bax, and Bcl-2. The MTT assay was used to determine IC_{50}, and the Chou-Talay method was used to determine the synergistic concentration of the combination treatment of diclofenac plus piperine and diclofenac plus *D*-limonene. Apoptosis detection, cell cycle arrest, reactive oxygen species production, and mitochondrial membrane potential were also investigated.

Results: Diclofenac, piperine, and *D*-limonene showed potent binding affinity for p53, Bax, and Bcl-2. Diclofenac plus piperine and diclofenac plus *D*-limonene enhanced the formation of reactive oxygen species, which also had an effect on the mitochondrial membrane's integrity and caused DNA fragmentation. Diclofenac plus piperine and diclofenac plus *D*-limonene arrested the cells in the sub-G_0 phase while drastically lowering the percentage of cells in the G_2/M phase. Furthermore, the elevated apoptosis in the combined therapy was confirmed by annexin V/propidium iodide staining.

Conclusions: The combined therapy prominently enhanced the anti-proliferative and apoptotic effects on MCF-7 cells compared with treatment with diclofenac, piperine, and *D*-limonene alone.

KEYWORDS: Breast cancer; Diclofenac sodium; Piperine; *D*-limonene; Reactive oxygen species; Cell cycle arrest; Apoptosis

1. Introduction

One of the foremost causes of death around the globe is cancer[1]. The number of new cases of cancer grew by 26.3%, from 18.7 million in 2010 to 23.6 million in 2019 worldwide[2]. Breast cancer is the most common malignancy in women and it is a complex disease, meaning diverse events may cause it. Although the condition is present everywhere, there are significant regional variations in the condition's occurrence, mortality, and survival rates. Many aspects may affect these variations, including population distribution, traditions and culture, genetics, and environment[3]. There are many chemotherapeutic agents in the market, and the main drawbacks are drug resistance as well as the toxicity of the agent to normal cells. Many drugs are being repurposed for the treatment of other diseases. A category of substances classified as non-steroidal anti-inflammatory drugs (NSAIDs) has anti-inflammatory, vasodilator, vasoconstrictor, analgesic, and antipyretic properties. They function by inhibiting cyclooxygenase as well as lysyl oxidase which are necessary for the biogenesis of prostaglandins[4].

Significance

Breast cancer is the prevalent cancer in women. Our study shows that diclofenac plus piperine and diclofenac plus *D*-limonene can enhance the anti-proliferative and apoptotic effects on MCF-7 cells compared with treatment with these drugs alone. Therefore, diclofenac combined with piperine or *D*-limonene could be further explored as a potential treatment for breast cancer, which needs to be evaluated in future *in vivo* and clinical studies.

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Eicosanoids that include prostaglandins, thromboxanes, leukotrienes, lipoxins, resolvins, and eoxins are associated with both inflammation and cancer and are produced from arachidonic acid by cyclooxygenase and lysyl oxidase[5]. Numerous studies have revealed an adverse relationship between the consumption of NSAIDs and the occurrence of many kinds of cancer, including mammary[6], lung[7], and gastric[8]. These findings have stimulated investigations on the potential anti-cancer effects of NSAIDs.

An *in vitro* experiment combining diclofenac and sorafenib was conducted on nine different melanoma cell lines[9]. A biological analysis was carried out to find effective pharmacological synergy combinations against melanoma cell lines that exhibit the foremost genetic causes of the condition (BRAF, NRAS, and CDKN2A). Irrespective of the genotype of the cell lines, sorafenib and diclofenac therapy was efficient. Another team of researchers evaluated the *in vitro* efficacy of sorafenib and diclofenac against hepatocellular carcinoma cell line HepG2 and showed that cell proliferation was considerably decreased at 50 µg/mL[10]. Piperine, a nutritious flavonoid from black and long pepper, has anti-inflammatory properties and also hinders the proliferation and dissemination of tumor cells[11] with reduced toxicity to normal cells.

The main bioactive food ingredient in citrus peel oil is *D*-limonene. *D*-limonene could inhibit or postpone the progression of several types of cancer, including lymphomas[12], breast[13], stomach[14], liver[15], as well as prostate cancer[16] in animal and cell culture models. It also exhibits various properties similar to many chemopreventive agents. Given that the mechanisms governing the occurrence and progression of many breast cancers are complex, *D*-limonene probably influences several anti-cancer pathways[17]. Such property is crucial for the advancement of drugs for the prevention and treatment of breast cancer. *D*-limonene is non-polar chemically, has high lipophilicity, and is prone to deposit in fatty tissues following oral ingestion, including the breast. Therefore, *D*-limonene can be explored as a chemopreventive agent against particular tumors, especially tumors developing in regions of elevated adiposity like the breast, given the pre-clinical data and its structural characteristics[18].

Earlier studies claimed that diclofenac[19], piperine[20], and *D*-limonene[15] had an anti-proliferative effect on cancer cells whether used separately or in combination with anti-cancer drugs. Moreover, in a study conducted by Chen *et al.*[21], piperine reduced the expression of the proteins Bcl-2, Bcl-xl, pPI3k, and pAkt while increasing the levels of Bax, Bad, Cyto C, cleaved PARP, and caspase-3. PI3K/Akt survival axis was inhibited in HCT 116 colon cancer cells as a result of diclofenac-induced dephosphorylation of PTEN, PDK, and Akt[22]. *D*-limonene increased Bax and cleaved PARP during treatment in a report published by Yu *et al.*[23], indicating that the mitochondria-mediated intrinsic death pathway may be crucial in the destruction of lung cancer cells caused by *D*-limonene, which is compatible with some of its significance in the therapy of many other malignancies. Nevertheless, no study has yet examined the combined anti-cancer properties of these two natural compounds and diclofenac. We hypothesized that using these drugs/compounds together would have a synergistic effect and lower the dosage and the side effects of diclofenac, such as an ulcer. In the present investigation, we examined the effects of diclofenac sodium, piperine, and *D*-limonene, as well as their combination on a breast cancer cell line.

2. Materials and methods

2.1. In silico study

2.1.1. Molecular docking

The mechanism of action of the ligands diclofenac, *D*-limonene, eugenol, piperine, and curcumin against significant proteins involved in the apoptotic signaling cascade was predicted using *in silico* docking analyses.

2.1.2. Protein structure preparation

To investigate the physicochemical properties of apoptotic proteins, cellular tumor antigen p53 (PDB: 1YCR), Bcl-2 (PDB: 2O21), and Bax (PDB: 1F16) will be docked. To assign the correct bond ordering and add any missing hydrogens, the selected chains of the proteins were modified. The sample orientations were used to maximize the H-bonds. A display of all polar hydrogens was made. Utilizing the software SPDBViewer, the protein structure was lastly energy minimized.

2.1.3. Ligand structure preparation

PubChem was employed to download the structures of the ligands diclofenac, *D*-limonene, and piperine. The files were retrieved in SDF format, which was easily convertible into PDB format using the SMILES translator for additional screening steps.

2.1.4. Platform used for docking and visualization

Using Auto Dock Vina on the PyRx platform, the best orientations of the ligands and target structures for diclofenac, curcumin, *D*-limonene, eugenol, and piperine with the highest binding affinities were examined (version4, The Scripps Research Institute, USA). The active sites of each protein-ligand complex, along with the high-affinity binding pockets they are associated with, as well as non-covalent interactions for potential ligands, were shown in the Discovery studio visualizer (version16.1.0.15350, Biovia, USA).

2.2. In vitro study

2.2.1. Chemicals

Piperine (Mol. Wt: 285.34) and *D*-limonene (Mol. Wt: 136.23) were purchased from HIMEDIA. A local drugstore provided the diclofenac sodium (Voveran® solution for Intramuscular injection 75 mg/mL) to test the commercially accessible medication, which patients use, and not the pure molecule. Extempore dilution of the solutions was done. SRL Chemicals Pvt. Ltd, Mumbai, India, supplied all solvents used in this study. Without additional purification, all the chemicals and reagents were utilized in their original form. The overall experiment was conducted with deionized water.

2.2.2. Cell culture and reagents

The human breast cancer MCF-7 cell line was procured from NCCS, Pune, India. Cells were maintained in DMEM high glucose medium along with 10% fetal bovine serum and 1% penicillin/ streptomycin combination in a humidified chamber in an atmosphere of 95% air and 5% CO_2 at 37℃.

2.2.3. Cell cytotoxicity analysis

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the cytotoxicity induced by diclofenac, piperine, and *D*-limonene individually, as well as diclofenac plus piperine and diclofenac plus *D*-limonene against cancer cells. For the assay, MCF-7 cells were seeded in a 96-well plate at a density of 10^4 cells per well and maintained at 37℃ for 24 h till it reached 80%-90% confluency. To examine the cytotoxicity of the compounds individually, the cells were treated with compounds in diverse doses (diclofenac: 50-500 μM; piperine: 100-500 μM; *D*-limonene: 100-500 μM) and incubated for 24 h at 37℃. After 24 h incubation, 100 μL of MTT solution [0.5 mg/mL of MTT in phosphate buffered saline (PBS)] was added to each of the wells and kept for 4 h of incubation. The formazan crystals developed due to the interaction of MTT and live cells were dissolved using dimethyl sulfoxide (DMSO), and the absorbance was recorded at 570 nm by a microplate reader. The cell viability percentage was calculated using the formula:

$$\text{Cell viability \%} = \frac{\text{OD value of samples}}{\text{OD value of control}} \times 100$$

2.2.4. Determination of combination index (CI)

CompuSyn software (Biosoft, Ferguson, MO) was used to analyze the interaction among the two or three molecules using the Chou-Talay approach[24]. This technique uses multiple drug-effect equations developed from an enzyme kinetics model with a CI. The CI values were acquired throughout a fractional cell death level range (Fa) of 0.05 to 0.95 (5%-95% cell kill). According to the Chou-Talay technique, CI<1 denotes synergism, CI=1 denotes additivity, and CI>1 denotes antagonism. Synergistic effects are the result of two drugs interacting in a way that increases the effects of either one or sometimes both drugs. A circumstance in which the combined effects of the two drugs result in the same outcome is referred to as an additive effect. When a drug interaction lessens the effectiveness of one or both medications, it is considered antagonistic.

2.2.5. Hemocompatibility assay

In this assay, we sought to ascertain whether or not diclofenac, *D*-limonene, piperine, diclofenac plus piperine, and diclofenac plus *D*-limonene protected the erythrocyte membrane from oxidative damage. The previously published protocol[25] was used to determine the hemocompatibility of the individual drug's IC_{50} (diclofenac, piperine, and *D*-limonene) concentrations and synergistic concentration of the drug combination. The heparinized blood collected from healthy rats was used to isolate red blood cells. The cells were then washed three times using HEPES buffer. 10^7 cells in 1 mL of suspension were placed per microfuge tube, and each sample was placed for a 30 min incubation at 37℃. After centrifuging the tubes at 4000 rpm for 15 min, the absorbance of the supernatant at 540 nm was determined. The percentage of hemolysis was estimated using the following calculation, with the lysis obtained with water being taken as 100%.

$$\text{Hemolysis \%} = \frac{A_{\text{sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \times 100$$

Where $A_{\text{positive control}}$ is the absorbance of the positive control (distilled water), $A_{\text{negative control}}$ is the absorbance of the negative control (the HEPES buffer mixed with blood sample) and A_{sample} is the absorbance of treated samples.

2.2.6. Detection of apoptosis by dual acridine orange/ ethidium bromide (AO/EB) fluorescent staining

Dual AO/EB fluorescent labeling can be utilized to observe apoptosis-related alterations in cell membranes under a fluorescent microscope. The cellular apoptosis in MCF-7 cells was detected using the dual AO/EB staining technique. The cells were treated with IC_{50} of each drug alone, and synergistic concentrations of drug combination. After the treatment, the cells were incubated for 24 h, the media were removed, and the plates were washed with PBS. AO/EB (100 μg/mL) was added, incubated for 20 min, and observed under the fluorescent microscope (Lawrence & Mayo, India).

2.2.7. Detection of mitochondrial membrane potential (MMP) by rhodamine 123 staining

A cationic dye called rhodamine 123 binds to MMP and releases

a significant amount of green fluorescence. Only active cells' mitochondrial membranes exhibit the accumulation of the dye. Rhodamine-123 fluorescent staining was used to examine the MMP level in the cells. The MCF-7 cells were seeded in 6-well plates, treated with IC_{50} concentrations of the drugs individually and synergistic concentrations of drug combination, and incubated for 24 h. After 24 h of incubation, the treated cells were incubated with rhodamine 123 for 30 min and inspected under the fluorescent microscope (Lawrence & Mayo, India).

2.2.8. Determination of intracellular reactive oxygen species (ROS) production using 2',7'–dichlorofluorescein diacetate (DCFH–DA)

Using the DCFH-DA fluorescent staining procedure proposed by Kim *et al.*[26], the level of intracellular ROS accumulation was examined. Briefly, MCF-7 cells were plated in 6-well plates, subjected to IC_{50} concentrations of each drug alone as well as the synergistic concentrations of drug combination, and then incubated for 24 h. After that, the treated cells were incubated for 10 min with DCFH-DA. ROS accumulation was observed using a fluorescence microscope at the emission wavelength of 530 nm and the excitation wavelength of 485 nm (Lawrence & Mayo, India).

2.2.9. DNA fragmentation analysis using 4',6–diamidine–2'–phenylindole dihydrochloride (DAPI)

DNA fragmentation is one of the best experiments to demonstrate the onset and progression of apoptosis. Briefly, MCF-7 cells were plated in 6-well plates, subjected to IC_{50} concentrations of each drug alone as well as the synergistic concentrations of drug combination, and then incubated for 24 h. Then, DAPI was added and incubated for 10 min at 37℃. After DAPI labeling, DNA fragmentation was examined at the excitation and emission wavelengths of 358 and 460 nm using a fluorescent microscope (Weswox Optik, India).

2.2.10. ROS quantification using flow cytometry

The IC_{50} concentrations of each drug alone and the synergistic concentrations of drug combination were applied to the cells after they were seeded in a six-well plate. Cells were trypsinized and washed 24 h later. The cells were then treated with 20 µM of DCFH-DA at 37℃ for 30 min in complete darkness. Flow cytometry was employed to measure the ROS levels, and the analysis software cytexpert was used to interpret the data (CytoFLEX, Beckman Coulter, USA).

2.2.11. Cell cycle analysis

In order to calculate the proportion of cells in each phase of the cell cycle, a flow cytometry device (CytoFLEX, Beckman Coulter, USA) was used to track the progression of the cell cycle. For cell cycle analysis, MCF-7 cells (2×10^5 cells/well) were seeded in 6 well plates for 24 h and then treated with the IC_{50} of each drug alone as well as the synergistic concentrations of drug combination. The treated cells were then trypsinized, washed with PBS, fixed with 70% ethanol, and kept at −20℃ overnight. The cells were centrifuged the next day at 4000-5000 rpm for 6-7 min, and the pellet was suspended in PBS containing RNase and PI (50 µg/mL). The findings of cell cycle analysis were obtained using a flow cytometer (CytoFLEX, Beckman Coulter, USA) at the wavelength of 488 nm.

2.2.12. Annexin V/PI for apoptosis detection

The evaluation of cell apoptosis was carried out using an annexin V/PI apoptosis detection kit (AAT Bioquest, 22839). MCF-7 cells were exposed to IC_{50} concentrations of each drug alone, and the synergistic concentrations of drug combination for 24 h at 37℃. Following manufacturer's instructions, the cells were harvested, twice-washed with PBS, resuspended in 1× annexin V binding buffer, and then incubated at 37℃ for 30 min. The fluorescence at annexin V-518 nm and PI-620 nm was recorded to measure the percentage of apoptosis using a flow cytometer (CytoFLEX, Beckman Coulter, USA).

2.3. Statistical analysis

In this study, each experiment was performed thrice. The statistically significant difference between the samples was examined by one way ANOVA followed by Dunnett's test using graph pad prism 8 software. P values<0.05 were considered statistically significant.

2.4. Ethical statement

This study was approved by Institutional Animal Ethical Committee (IAEC) (Approval number and date: VIT/IAEC/21/Sep22/13, September 2022).

3. Results

3.1. Molecular docking study

The molecular docking and Discovery studio visualizer v16.1.0.15350 data were used to evaluate the binding affinity of a protein-ligand complex and interpret the findings. Following the completion of the docking analysis, the van der Waals interaction, Pi-Pi stacking interaction, and the conventional hydrogen bond interaction as well as the binding affinity results are given in Figure 1. The binding energy of curcumin against Bcl-2, Bax and p53 was −7.9, −1.3 and −5.2 kcal/mol, respectively, and the binding energy

of eugenol against the proteins Bcl-2, Bax and p53 was −5.7, −5.0 and −3.8 kcal/mol, respectively. Diclofenac showed the best binding energy against the protein Bax, piperine showed the highest binding energy against Bcl-2, whereas *D*-limonene showed negative binding energy against all three targeted proteins. Positive free energy levels show that the interactions or linkages were initiated by energy and that binding did not take place automatically. All of the values were negative, meaning that binding occurred spontaneously and the values are given in Table 1.

Table 1. Binding energies of diclofenac, piperine and *D*-limonene for p53, Bax and Bcl-2.

Protein	PDB ID	Ligand	Binding energy (kcal/mol)
p53	1YCR	Diclofenac	−4.6
		D-limonene	−4.0
		Piperine	−5.1
Bax	1F16	Diclofenac	−6.0
		D-limonene	−5.4
		Piperine	−3.7
Bcl-2	2O21	Diclofenac	−7.1
		D-limonene	−5.8
		Piperine	−7.5

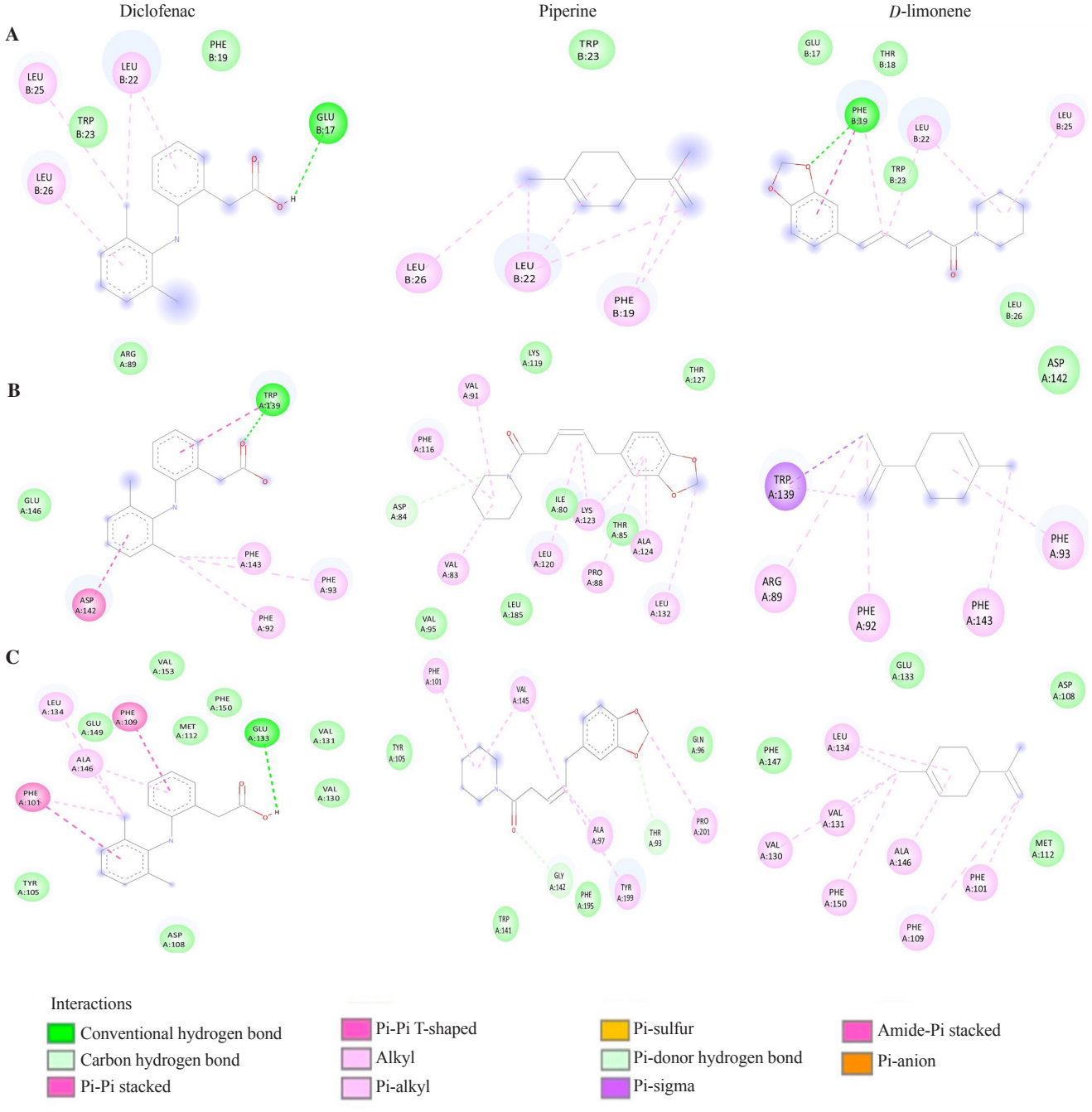

Figure 1. 2D representation of the interaction between ligand molecules. Diclofenac, piperine, *D*-limonene with the target proteins (A) p53, (B) Bax and (C) Bcl-2.

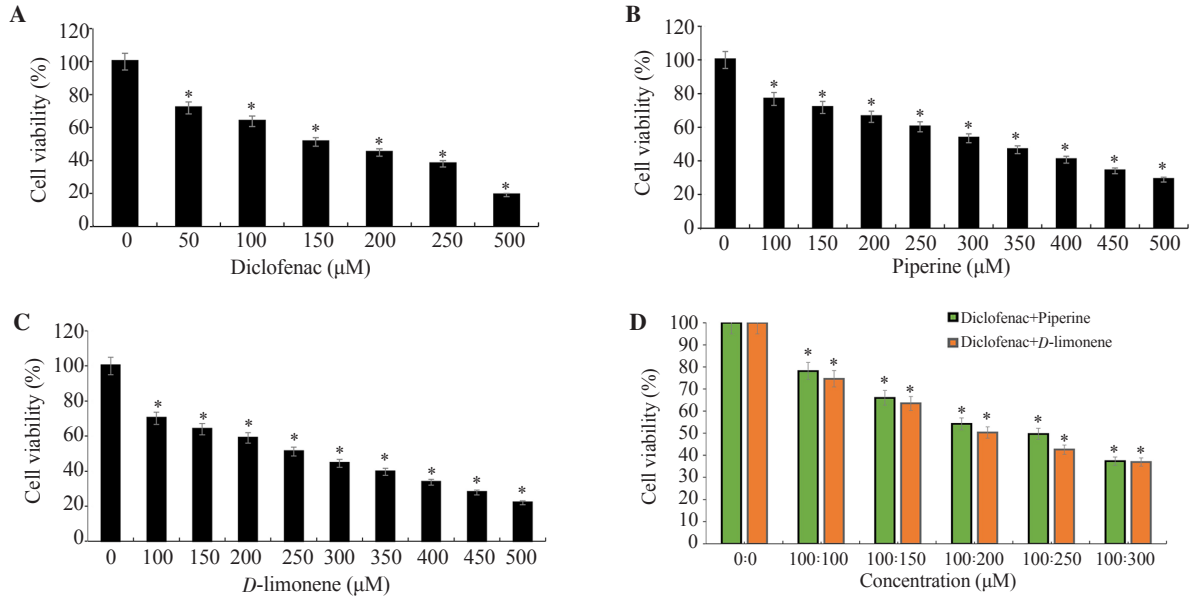

Figure 2. Cytotoxic effect of diclofenac (A), piperine (B), *D*-limonene (C), and diclofenac plus piperine and diclofenac plus *D*-limonene (D) on MCF-7 cells. $^{*}P<0.001$ compared with the control group.

3.2. Combinational effect of piperine and D–limonene with diclofenac on MCF–7 cell viability

The effects of diclofenac, piperine, and *D*-limonene against MCF-7 cell line are represented in Figure 2. The IC_{50} of diclofenac, piperine, and *D*-limonene was estimated to be 150 μM, 300 μM, and 250 μM, respectively. Treatment with each drug alone as well as their combination showed a dose-dependent anti-proliferative effect against MCF-7 cells. In addition, a synergistic dose of diclofenac plus piperine (100 μM+250 μM) with a CI=0.830 and diclofenac plus *D*-limonene (100 μM+200 μM) with a CI=0.896 showed IC_{50} value and was used for further *in vitro* studies, according to estimations of the combined effects of diclofenac with piperine or *D*-limonene (Figure 3).

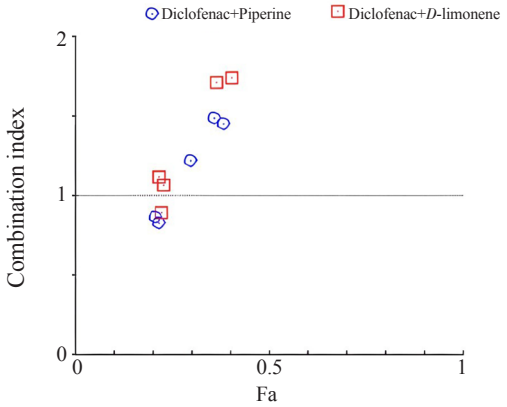

Figure 3. Combination index of diclofenac plus piperine as well as diclofenac plus *D*-limonene.

3.3. Hemocompatibility assay

The percentages of hemolysis for diclofenac, piperine, *D*-limonene, diclofenac plus piperine and diclofenac plus *D*-limonene were (2.080±0.072)%, (1.400±0.054)%, (1.200±0.064)%, (1.980±0.067)% and (1.090±0.051)%, respectively. Diclofenac plus piperine and diclofenac plus *D*-limonene showed lesser hemolysis when compared to the hemolysis percentage of diclofenac alone.

3.4. Combinational effect of piperine and D–limonene with diclofenac on apoptosis induction in MCF–7 cells

The induction of apoptosis in MCF-7 cells was verified by AO/EB staining. Control cells displayed green fluorescence, which indicates that they are alive and free of apoptosis. Cells treated with diclofenac, piperine, and *D*-limonene displayed mixed green (live cells), yellow-green (early apoptotic cells), and orange (late apoptotic cells) fluorescence at the IC_{50} concentrations. More late apoptotic cells and fewer early apoptotic cells were seen in diclofenac plus piperine and diclofenac plus *D*-limonene treated cells (Figure 4A).

3.5. Combinational effect of piperine and D–limonene with diclofenac on MMP in MCF–7 cells

As shown in Figure 4B, compared to diclofenac plus piperine and diclofenac plus *D*-limonene, a small percentage of cells exhibited impaired membrane integrity of the mitochondria when the cells were treated with IC_{50} concentrations of diclofenac, piperine, and *D*-limonene alone. This outcome demonstrates the superior efficacy of a combination treatment over a single agent.

3.6. Combinational effect of piperine and D–limonene with diclofenac on DNA fragmentation

Figure 4C demonstrates that compared to the cells treated with the IC_{50} concentrations of diclofenac, piperine, and *D*-limonene individually, the cells treated with diclofenac plus piperine or diclofenac plus *D*-limonene displayed higher DNA fragmentation, indicating that the cell may experience an apoptotic mechanism of cell death that commences with the fragmentation of DNA.

3.7. Qualitative and quantitative ROS analysis using flow cytometry

To qualitatively determine whether apoptosis induced by the combination treatment (diclofenac plus piperine and diclofenac plus *D*-limonene) was associated with ROS-mediated oxidative stress, intracellular ROS production was analyzed using DCFH-DA fluorescence assay. As indicated in Figure 5, treatment with IC_{50} concentrations of diclofenac, piperine, and *D*-limonene alone generated significantly less ROS when compared to diclofenac plus piperine and diclofenac plus *D*-limonene, indicating the effectiveness of combinatorial drug treatment over a single drug molecule.

To quantitatively evaluate the generation of ROS, flow cytometry analysis was performed. The elevated ROS levels reveal the greater fluorescence intensity. As shown in Figure 5, the ROS percentage in control cells was 22.37%, whereas, the ROS percentage of the cells treated with IC_{50} concentrations of diclofenac, piperine, and *D*-limonene was 65.87%, 61.05%, and 68.68%. Moreover, the percentage of ROS was 72.55% and 88.03% in diclofenac plus piperine and diclofenac plus *D*-limonene treated cells, respectively.

3.8. Apoptosis detection using flow cytometry

Figure 6 shows that treatment with diclofenac plus piperine and diclofenac plus *D*-limonene significantly induced apoptosis in MCF-7 cells (early apoptosis: 62.65% and 55.56%; late apoptosis: 5.54% and 6.04%) compared with treatment with the IC_{50} concentration of diclofenac, piperine, and *D*-limonene alone (early apoptosis: 18.85%, 8.67% and 36.85%, late apoptosis: 2.44%, 0.47%, 2.89%).

3.9. Cell cycle analysis using flow cytometry

The combinational drug diclofenac plus piperine and diclofenac plus *D*-limonene dramatically increased the percentage of cells in the Sub-G_0 phase while significantly decreasing the percentage of cells in the G_2/M phases, compared with treatment with each drug alone (Figure 7).

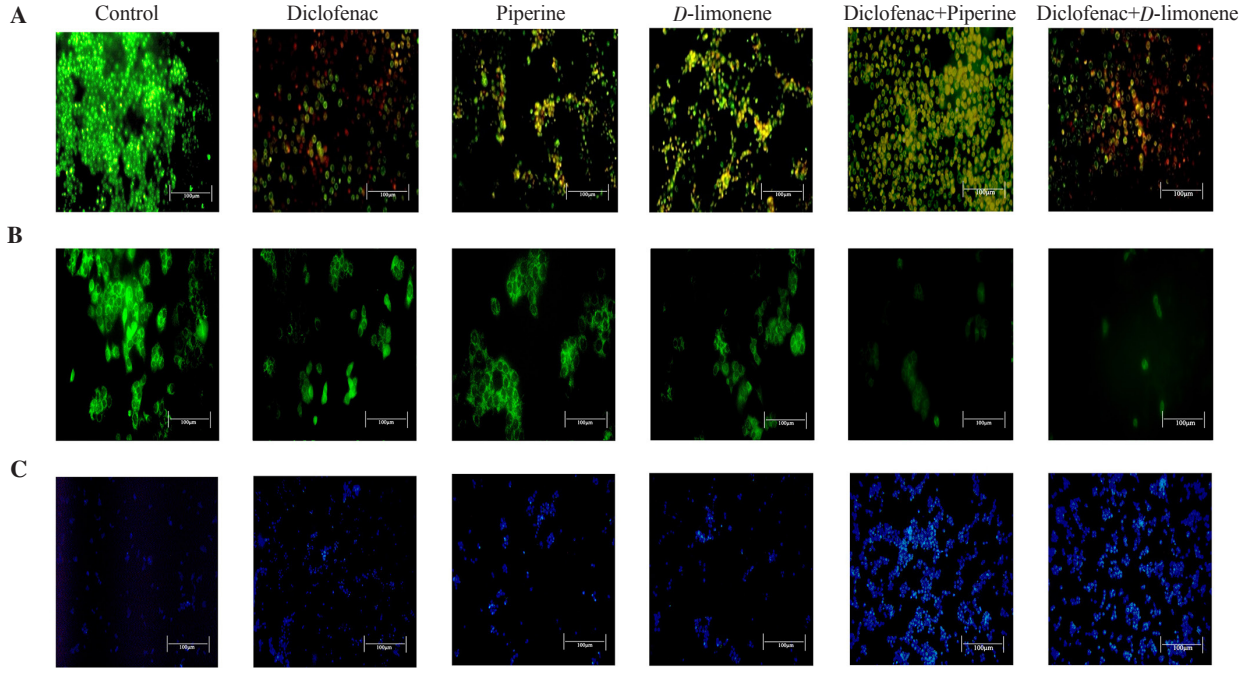

Figure 4. (A) Apoptosis detection using AO/EB staining. Apoptosis of the control and treated cells was determined by a fluorescent microscope using AO/EB stain. (B) Mitochondrial membrane potential detection. The mitochondrial membrane potential of the control and the treated cells was determined by a fluorescent microscope using rhodamine-123 stain. (C) DNA fragmentation detection. The fragments of DNA in the control and treated cells were determined by a fluorescent microscope using DAPI stain. All images were taken at 20× magnification.

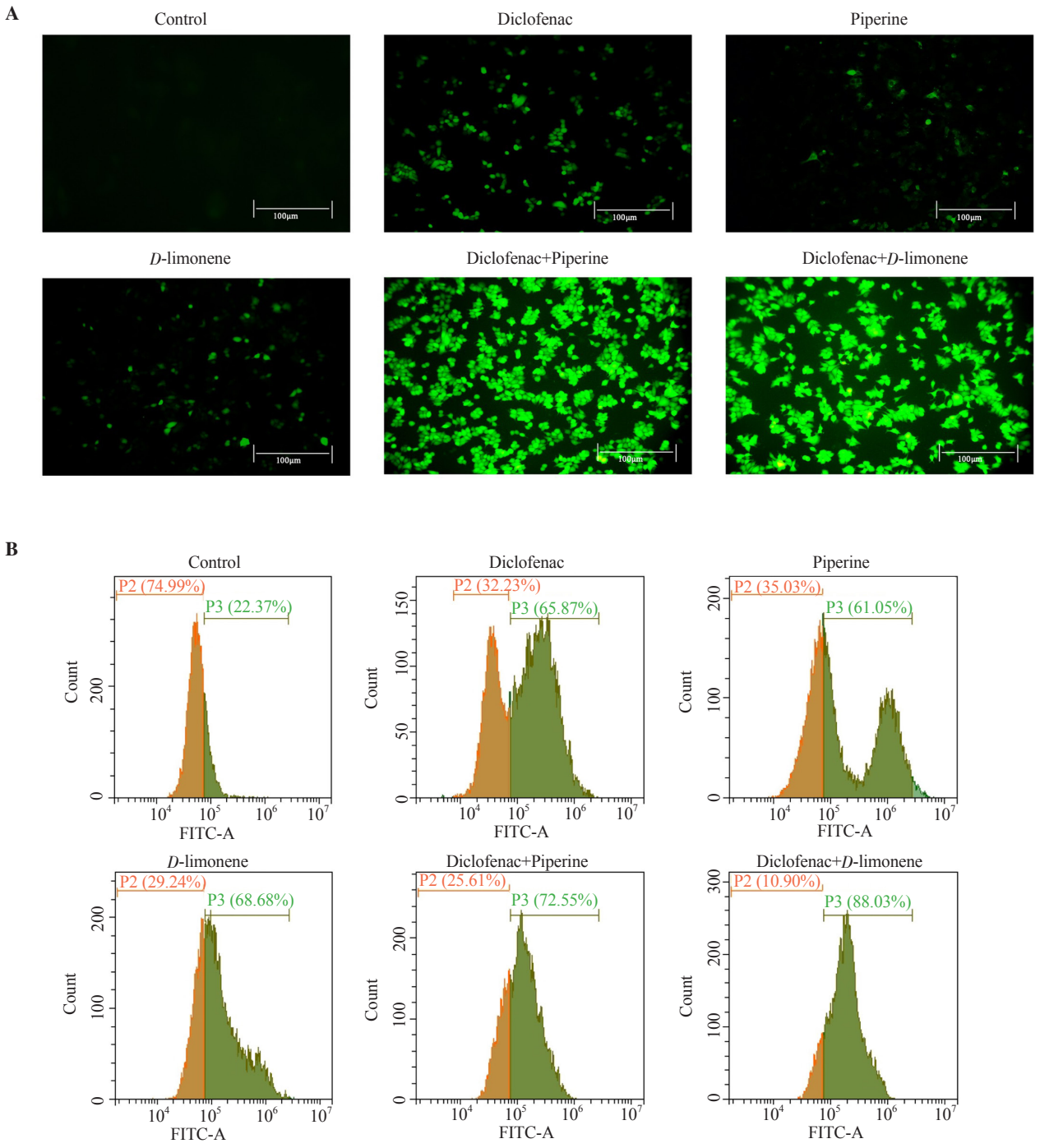

Figure 5. Qualitative and quantitative analysis of ROS. (A) ROS production of the control and treated cells was determined by a fluorescent microscope using DCFH-DA dye. (B) Flow cytometric analysis of ROS production from MCF-7 cells treated with each drug alone or combination drugs. All images were taken at 20× magnification.

4. Discussion

The main drawbacks of the most widely used chemotherapeutic medications include cancer relapse, drug resistance, and harmful effects on normal tissues, which might limit the use of anticancer medications and reduce the life expectancy of patients[27].

The hunt for a novel and potential anticancer drug with enhanced efficacy and fewer side effects is ongoing to address the issues with current treatment. Typically, combined chemopreventive strategies are preferred over single-agent chemoprevention. To attain optimum chemopreventive effectiveness with minimal toxicity, a combination method uses various chemopreventive drugs at low dosages[28]. Piperine's cancer-preventive effects against a variety of cancer-causing chemicals, including 7,12-dimethyl benz(a)anthracene and benzo(a)pyrene, highlight its ability as a chemopreventive molecule[29,30]. *D*-limonene and structural analogues have exhibited substantial chemopreventive benefits in animal models of lymphomas, mammary, gastric, hepatic, and lung malignancies[12,31].

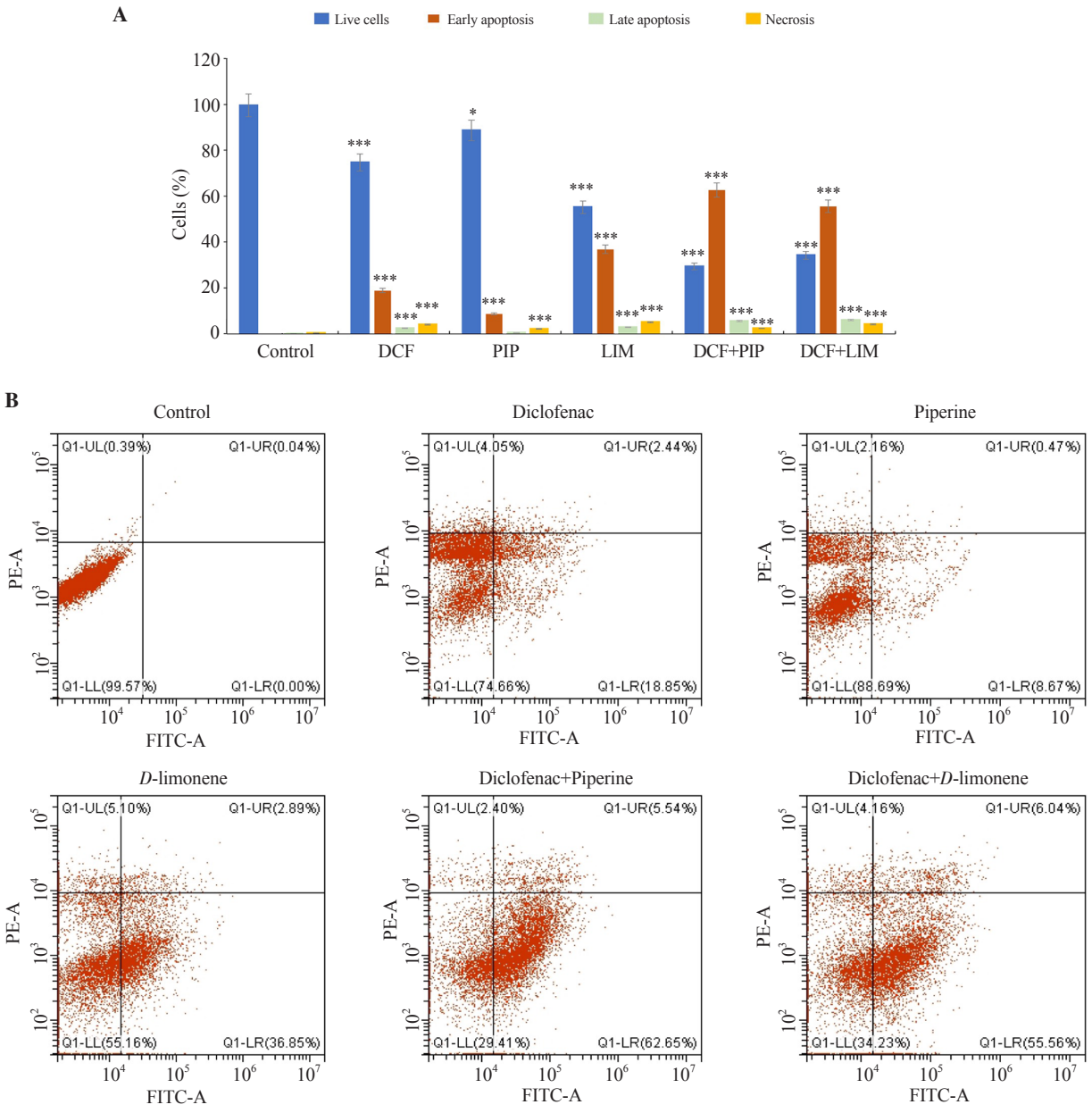

Figure 6. Apoptosis detection using flow cytometry. The apoptosis of MCF-7 cells after combinational treatment was determined by flow cytometry using Annexin V/PI. A: A bar graph of flow cytometric analysis. B: Flow cytometric analysis of MCF-7 cells treated with each drug alone or combination drugs. $^{*}P<0.05$ and $^{***}P<0.001$ compared with the control group. DCF: Diclofenac; PIP: Piperine; LIM: *D*-limonene.

The preclinical data is most favourable for mammary carcinogenesis and suggests a potential chemopreventive role.

Diclofenac is a nonsteroidal anti-inflammatory medication that is widely used. It has a well-established position in oncological medicine as a topical treatment for actinic keratosis, which is usually regarded as pre-cancerous lesions. The anti-proliferative impact of diclofenac has been studied in many cancers such as neuroblastoma[32], ovarian cancer[33], pancreatic cancer[19], melanoma[9], hepatocellular carcinoma[10], and prostate cancer[34].

The results of the molecular docking demonstrate that the primary apoptotic proteins, p53, Bax, and Bcl-2, can be bound with diclofenac, piperine, and *D*-limonene with adequate affinity. Anticipating the most probable binding poses of a drug or ligand with a particular three-dimensional protein structure is the aim of ligand-protein docking. Molecular docking has developed into a powerful and affordable method for finding novel lead molecules. Based on these findings, all three of these compounds were subsequently selected for the *in vitro* analysis. The proliferation of MCF-7 cells was substantially inhibited after administering a combination of diclofenac and piperine (100 μM+250 μM) or and *D*-limonene (100 μM+200 μM) compared with treatment with these drugs individually, and this concentration of combinational drug

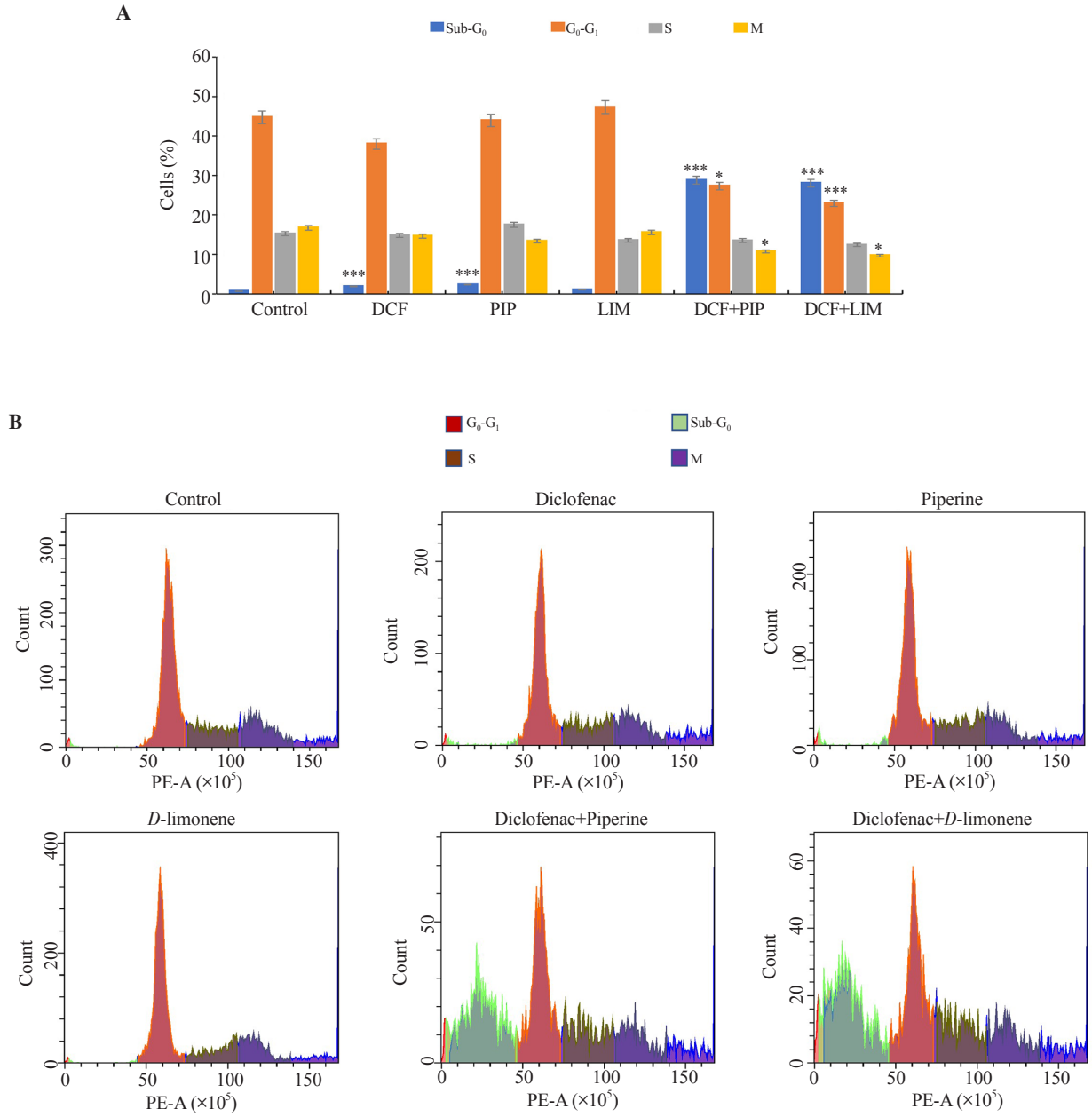

Figure 7. Cell cycle analysis of MCF-7 after combinational treatment (diclofenac plus piperine and diclofenac plus *D*-limonene) by flow cytometry. A: A bar graph of flow cytometric analysis. B: Flow cytometric analysis of MCF-7 cells treated with each drug alone or combination drugs. $^{*}P<0.05$ and $^{***}P<0.001$ compared with the control group.

showed synergism according to Chou-Talay method[24]. The main goal of this research was to determine whether a pharmacological combination could serve as a more effective therapeutic agent than a drug taken alone. According to the cytotoxicity study results, the combination of drugs reduced the number of cells in a dose-responsive manner and to a more considerable extent than each drug individually. This was also confirmed using AO/EB dual staining method. The results showed that diclofenac combined with piperine or *D*-limonene significantly induced apoptosis than application of each drug alone in MCF-7 cells. The majority of cells found within the human body are erythrocytes, and oxidation damages the lipids and proteins in the erythrocyte membrane during hemolysis[35]. In our study, the combination of diclofenac with piperine and *D*-limonene showed 1.98% and 1.09% hemolysis, respectively and the results indicate that both individual and combinational compounds showed a negligible percentage of hemolysis, indicating that they are not hemolytic and therefore compatible with blood cells.

The principal intracellular activity following the activation of apoptosis occurs in the mitochondria, which are the prominent location of ROS production in cells[36]. Due to the substantial inside-negative transmembrane potential of the mitochondria, positively charged and lipophilic rhodamine123 distributes into the mitochondria over the period. In isolated mitochondria or

undamaged cells, this mitochondrial localization has indeed been employed as an indication of MMP[37]. A reduction in MMP is associated with an increase in ROS levels. The potential of the mitochondrial membrane was evaluated as we assessed the likelihood that the drugs used in our study may affect mitochondrial function. Since rhodamine's fluorescence declines when the MMP is reduced, it was used to monitor changes in MMP. In our study, compared to diclofenac combined with piperine and *D*-limonene, only a small percentage of cells exhibited impaired membrane integrity of the mitochondria when the cells were treated with IC_{50} concentrations of diclofenac, piperine, and *D*-limonene alone. This outcome demonstrates the superior efficacy of a combination treatment over a single agent. Fragmentation of the genomic DNA is a standard indicator for the initiation of apoptosis. The toxicity of the compounds to the cells is shown using the MTT assay. Even though cell death demonstrated the cytotoxicity of the compounds at various doses, it is crucial to comprehend the process of death before considering the compound for the treatment of cancer.

ROS generation is associated with cancer cell survival and progression. Alteration in this redox balance and dysregulated redox signaling may lead to cytotoxicity in cancer cells. To qualitatively and quantitatively determine whether apoptosis induced by diclofenac plus piperine and diclofenac plus *D*-limonene was associated with ROS-mediated oxidative stress, intracellular ROS production was analyzed using DCFH-DA fluorescence assay. As a result of intracellular esterases cleaving DCFH-DA, H2DCF, a non-fluorescent molecule that accumulates inside cells, is produced. DCF is then oxidised to produce a highly fluorescent product[38]. In a study conducted by Jafri *et al.*[39], the HeLa cells treated with 50 μM and 100 μM of piperine increased ROS generation up to 19.62% and 31.12%, respectively. In a study conducted by Rabi *et al.*[16], increased ROS levels play a role in cell death of prostate cancer, which was induced by a single treatment with docetaxel alone (1.9 nM) or a combination of 0.02 mM *D*-limonene. In research conducted by Li *et al.*[40], diclofenac-treated human corneal epithelial cells showed higher levels of intracellular ROS. In our study, the qualitative and quantitative results indicated that diclofenac plus piperine and diclofenac plus *D*-limonene remarkably enhanced ROS production. Moreover, the combined treatment demonstrated higher DNA fragmentation.

According to Chen *et al.*[21], the percent of apoptotic cells increases dose-dependently following piperine treatment of SNU-16 cells. At 150 μM, apoptotic cells in both the early and late phases increased noticeably, and the overall percentage of apoptotic cells reached 50.6%. *D*-limonene caused a dose-dependent activation of apoptosis in LS174T cells in a study by Jia *et al.*[41] (apoptosis rates at 0.5 μM/L, 1.6 μM/L, and 3.2 μM/L were 5%, 12%, and >15%, respectively). In a research work conducted by Poku *et al.*[42], extremely low levels of apoptosis were found in A549 cells treated with diclofenac alone, but a rise in apoptosis was seen in A549 cells co-treated with 10 μM of docosahexaenoic acid and diclofenac. In the present study, diclofenac plus piperine and diclofenac plus *D*-limonene induced more apoptosis than treatment with each drug alone.

The cell cycle is an intricate and complicated process, and its dysregulation may cause aberrant metabolism and growth. By targeting certain proteins, several anti-cancer medications prevent the passage of cells from one phase of the cycle to the next, causing a build-up of tumor cells at a given stage. Cell cycle arrest inhibits cancer cells from growing into tumors and spreading to other regions of the body[43]. Previous studies showed that piperine increased the sub-G_1 phase of the multidrug resistance cells, Lucena-1, and FEPS[44]. Zhang *et al.*[45] also revealed that *D*-limonene increased the G_0-G_1 phase of the human gastric carcinoma cell line MGC803. The number of human corneal epithelial cells in the S phase considerably increased in a time-dependent manner following treatment with 0.05% diclofenac sodium[40]. In this study, diclofenac plus piperine and diclofenac plus *D*-limonene dramatically increased the percentage of cells in the sub-G_0 phase while significantly decreasing the percentage of cells in the G_2/M phase.

In conclusion, diclofenac plus piperine and diclofenac plus *D*-limonene showed anti-proliferative effects against MCF-7 cells. The combination of these drugs also increased ROS generation and decreased MMP of MCF7 cells, which resulted in cell cycle arrest mostly in the sub-G_0 or G_1 phase and apoptosis. Diclofenac combined with piperine or *D*-limonene showed better anticancer efficacy against breast cancer cells than treatment with these drugs alone. However, further *in vivo* studies need to be conducted to verify its application. The expression of proteins that are largely involved in the apoptotic pathway also needs to be examined using RT-PCR and Western blotting analysis in the future in order to decipher the antiproliferative mechanism of these compounds.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

SS contributed to the conception and design, acquisition of data, drafting of the article, and final approval of the manuscript. GKM contributed to the analysis of data, critical revision of the manuscript content, and final approval of the manuscript.

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