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Evaluation of anticancer efficacy of umbelliferone with or without piperine

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Abstract
Phytocompounds are pleotropic in their function and preferentially target cancer cells without altering normal cells. In the present study, MTT [3-(4, 5- dimethylthiazolyl- 2)-2, 5- diphenyltetrazolium bromide] assay was conducted on MCF-7 and MDA-MB-231 cell lines at 48h incubation to determine percent cell viability. The IC_{50} values of umbelliferone and piperine for MCF-7 were 15.56 and 17.83µM, respectively. Likewise, IC_{50} values of umbelliferone and piperine for MDA-MB-231 were 10.31 and 14.28 µM, respectively. A combination of umbelliferone (IC_{25}) and piperine (IC_{25}) on MDA-MB-231 and MCF-7 showed percent cell viability of 8.24±0.01 and 15.12±0.01, respectively. This study suggests that the cytotoxicity of umbelliferone and piperine was dose-dependent. The *in vitro* cytotoxicity potential in terms of percent cell viability, early apoptosis, late apoptosis and G2M phase arrest of umbelliferone alone and in combination with piperine proved to be more effective on MDA-MB-231 cells.

Keywords: Umbelliferae, piperine, cytotoxicity, apoptosis, cell cycle arrest, MCF-7, MDA-MB-231

1. Introduction
Cancer is a multistage process and is the second leading cause of death after cardiovascular disease [1]. Although the mortality rate has declined with early detection [7], but the recurrence and metastases remain the leading causes of death [2, 3]. A triple-negative breast cancer (TNBC) is the most aggressive, difficult to treat, metastatic, poor prognosis [4, 5] and resistant to several anti-cancer agents [6]. Additionally, the development of multidrug resistance (MDR) is a major drawback to cancer chemotherapy which can reduce therapeutic efficacy and decreases the survival rates [7, 8, 9]. Therefore, new therapeutic agents with better therapeutic efficacy and safety profile are critically needed to improve the current treatment modalities [10, 11].
The umbelliferone is a 7-hydroxycoumarin, widely found in plants and is benzopyrone in nature. It exhibits various pharmacological activities against conditions related to oxidative stress such as inflammation, degenerative diseases, cancer and microbial infections [12]. It exerts anticancer effect against colon cancer [13], laryngeal cancer [14], hepatocellular carcinoma [15]. The piperine is a major alkaloid constituent of *Piper* sp belonging to the Piperaceae family. It produces various pharmacological activities such as anti-inflammatory, anti-cancer, antibacterial, immunosuppressive, anticonvulsant, analgesic and antiparasitic activities [16, 17, 18]. Hence, this study was aimed to evaluate the anticancer efficacy of umbelliferone with or without piperine in terms of cytotoxicity, apoptosis and cell cycle arrest in most commonly studied breast cancer cell lines *viz*, MCF-7 and MDA-MB-231.

2. Materials and Methods
2.1 Materials
Umbelliferone (Sigma, #H24003), Piperine (Sigma, #P49007), Dimethly sulfoxide (Sigma, #D8418) were purchased from Sigma-Aldrich Chemical Co. Ltd. FITC-annexin V (BD Biosciences, #556547), 10X annexin V binding buffer (BD Biosciences, #556547), propidium iodide (BD Biosciences, #556547), Dulbecco's Modified Eagle's Medium (GE Healthcare Life Sciences, #SH30243.01), Dulbecco's phosphate-buffered saline (GE Healthcare Life Sciences, #SH30028.02). Trypan blue, absolute ethanol, penicillin, streptomycin, amphotericin, fetal bovine serum, trypsin, paraformaldehyde (PFA), Triton-X 100, RN*ase*

(HiMedia Labs, Mumbai, India) and other reagents of analytical grade were also used in the study.

2.2 Cell lines and culture conditions

In vitro cytotoxicity study was carried out on breast cancer cell lines (MCF-7) and MDA-MB-231 cell lines (National Centre for Cell Science, Pune, India). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (4.5g/L Glucose), supplemented with L-glutamine (4mM), 10% heat-inactivated fetal bovine serum (FBS), streptomycin 1000µg/mL, penicillin 10,000 units/mL and Amphotericin-B solution 25µg/mL. The cells were incubated at 37^0C in a humidified atmosphere of 95% air and 5% CO_2. Following 24h of incubation, the adherent cells were detached using trypsin-EDTA solution 1X/0.25%. The cell count was carried out using the Luna automated cell counter (Logos Biosystems, USA) based on the trypan blue dye exclusion method.

2.3 Determination of cytotoxicity

The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was carried out on breast cancer cell lines *viz*., MCF-7 and MDA-MB-231 to determine the cytotoxicity of umbelliferone, piperine, and combination of umbelliferone with piperine. Cell suspension of 200µL was seeded in 96-well microplates (Corning®, USA) at a density of 20,000 cells/well and incubated in 5% CO_2 incubator at 37°C for 24h, after which the cells were exposed to a graded concentration (MCF-7 & MDA-MB-231: 3.125, 6.25, 12.5, 25, 50 and 100µM) of umbelliferone and piperine followed by incubation for 48h. Later media was removed from all treated and vehicle control (1% DMSO) cells and replaced with fresh media containing MTT (0.5 mg/ml) and were re-incubated for 3 hours at 37°C. MTT solution was removed and 100µL of DMSO was added to dissolve the insoluble formazan crystals formed within mitochondria of viable cells. The plate was incubated with DMSO for 5min with gentle shaking. The cell viability was determined by measuring the absorbance on a microplate reader (ELx800, BioTek, USA) at λmax 570 nm. The concentration of umbelliferone and piperine that resulted in 50% inhibition of cell growth was calculated as the half-maximal inhibitory concentration (IC_{50}) by constructing a dose-response curve. The IC_{50} for each cell line was determined by a linear curve fit generated after obtaining dose-response to six different concentrations of umbelliferone and piperine. The cell viability percentages (y-axis) were plotted against increasing concentrations of umbelliferone and piperine on the x-axis. IC_{50} values were estimated by using the linear regression equation

$$y = mx + c;$$

Where 50 is substituted for y, yielding x as the IC50 value.

$$\text{Cell Viability (\%)} = \frac{\text{Number of cells treated with the test item}}{\text{Number of untreated cells}} X100$$

Later, the cell viability percent for the combination of umbelliferone (IC_{25}) and piperine (IC_{25}) on MCF-7 and MDA-MB-231 cell lines was carried out at 48h incubation.

2.4 Analysis of apoptosis and necrosis using Annexin V-FITC/ PI staining by flow cytometry

The cell death analysis in MDA-MB-231 was performed using annexin V-FITC (Fluorescein is othiocyanate) / PI (propidium iodide) assay with a flow cytometer (BD Biosciences FACS Calibur, USA). The cells were cultured in 6-well plates for 24h at 37^0C in 5% CO_2 humidified atmosphere and were later exposed to umbelliferone and piperine at their respective IC_{50} and combination of umbelliferone (IC_{25}) and piperine (IC_{25}) for 48h. After incubation, cells were harvested and washed once with 1X DPBS and once with 1X annexin V binding buffer (0.1M HEPES/ NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$). The cells were then suspended in binding buffer at $0.5X10^6$ cells/mL concentration and stained with 5µL annexin V-FITC. This was followed by an incubation period of 10-15 min at room temperature (RT) in dark. After incubation, the cells were washed with binding buffer and fixed with 4% PFA-paraformaldehyde, 0.1% Triton-X 100 for 10-15 min at RT. The cells were washed with binding buffer and subjected to 0.25-0.5 mg/mL RN*ase* treatment for 20 min at 37^0C. Again, the cells were washed, suspended in binding buffer and stained with 5µL of PI and incubated for 10-15 min at RT. Later, the cells were suspended in 400µL of 1X annexin-V binding buffer and the apoptotic/necrotic populations were analyzed by flow cytometry using Fluorescence channel-1 (FL1) and Fluorescence channel-2 (FL2).

2.5 Analysis of cell cycle by flow cytometry

The MDA-MB-231 cells were cultured in 6-well plates for 24h at 37^0C in 5% CO_2 humidified atmosphere and were exposed to umbelliferone and piperine at their respective IC_{50} and combination of umbelliferone (IC_{25}) and piperine (IC_{25}) for 48h. The cells were harvested and washed once with 1X DPBS. The cells were then suspended in 100µL binding buffer at $1X10^6$ cells/mL concentration. Fixation was carried out by adding 0.7mL chilled 70% absolute ethanol drop wise with gentle overtaxing. Later, cells were fixed for 30 min at 4^0C, centrifuged at 2500 rpm and washed twice with 1X DPBS. The cells were then treated with 0.2 mg/mL R N*ase* for 20 min at 37^0C, followed by staining with 50 µg/mL PI for 5-10 min in dark. The cell cycle was then analyzed by flow cytometry using Fluorescence channel-2 (FL2).

2.6 Statistical analysis

The data of *in vitro* cytotoxicity studies were subjected to linear regression analysis to obtain dose-response curves and IC_{50} values. All the values were expressed as Mean± SD (Graph Pad Prism, Version 5). Apoptosis/necrosis stages and cell cycle stages were analyzed by using BD Cell Quest™ Pro software, Version 6 and difference in the percentage of the population between vehicle control and treatment groups were calculated based on the statistical data generated by the system (BD Biosciences FACS Calibur, USA). The *in vitro* cell culture assays such as MTT was analyzed by student *t*-test and apoptosis-necrosis and cell cycle were analyzed by one-way ANOVA followed by post hoc Tukey's multiple comparison test. All the values were expressed as Mean ± SD (Graph Pad Prism, version 5).

3. Results and Discussion

3.1 *In vitro* cytotoxicity assay

The cytotoxicity of umbelliferone and piperine on both MCF-7 and MDA-MB-231 cell lines was dose-dependent (Fig.1a and 1b, respectively). The IC_{50} values of umbelliferone and piperine for MCF-7 were 15.56 and 17.83µM, respectively. Likewise, IC_{50} values of umbelliferone and piperine for

MDA-MB-231 were 10.31 and 14.28 µM, respectively. A combination of umbelliferone (IC_{25}) and piperine (IC_{25}) on MDA-MB-231 and MCF-7 showed percent cell viability of 8.24±0.01 and 15.12±0.01, respectively (Fig.2a and 2b, respectively). Based on IC_{50} values and percent cell viability observed in MTT assay it was concluded that MDA-MB-231 cells were more sensitive than MCF-7 cell lines to the test compounds. Hence, for further studies, MDA-MB-231 cells were selected for the evaluation of apoptosis/necrosis and cell cycle arrest.

The MDA-MB-231 cells upon treatment with umbelliferone, piperine at their respective IC_{50} concentration and combination of umbelliferone + piperine at their respective IC_{25} concentration for 48h incubation showed significantly ($p< 0.0001$) increase in cytotoxicity potential in terms of early and late apoptosis and G2/M phase arrest, (Fig. 3, 4, 5, 6 and Table 1, 2) by flow cytometry.

In conclusion, the current study revealed that umbelliferone induced cytotoxicity by triggering apoptosis and G2M phase arrest in triple-negative breast cancer cells. Further, a combination of umbelliferone with piperine produced an enhanced anticancer effect in terms of percent cell viability, early and late apoptosis and cell cycle arrest. The anticancer effect of piperine on TNBC cells could be due to down regulation of G1-associated (cyclin D3, CDK4, E2F-1) and G2-associated (cyclin B, CDK1, Cdc25C) proteins, as well as an induction of p^{21} [19] and also apoptosis *via* the mitochondrial pathway [19, 20] which might have additionally contributed to the enhanced anticancer effect of the combination. Thus, the findings of our study may provide a basis for the identification of the precise mechanism of action of umbelliferone on triple-negative breast cancer. Furthermore, a combination of umbelliferone with piperine has a wide scope for application in the treatment of triple-negative breast cancer.

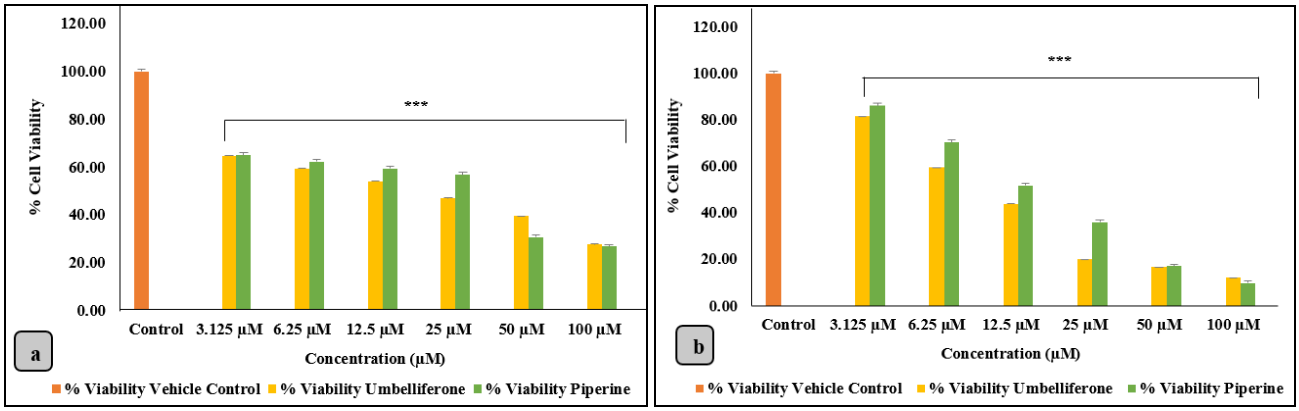


Fig 1: Histogram depiction of dose dependent cytotoxicity data of umbelliferone and piperine as determined by MTT assay on (a) MCF- 7 and (b) MDA-MB-231 cell lines. Values are Mean± SD, n=3, (***p<0.0001)

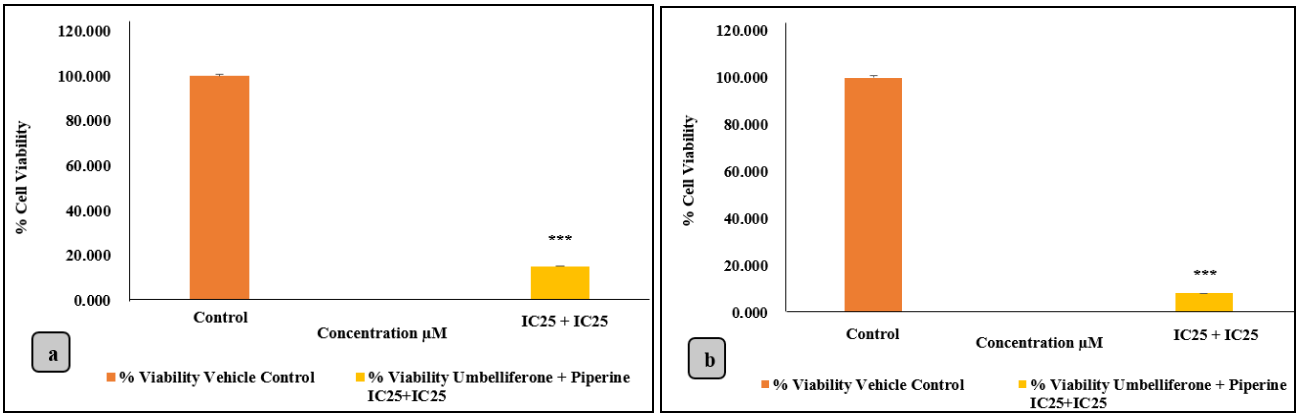
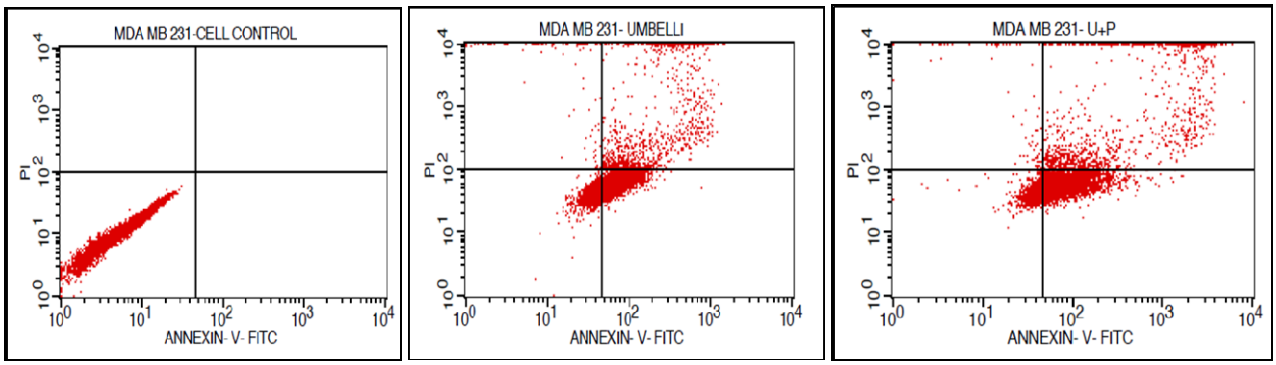


Fig 2: Bar graph depiction of cytotoxicity data of combination studies (umbelliferone IC25 and piperine IC25) as determined by MTT assay on (a) MCF- 7 and (b) MDA-MB- 231 cell lines. Values are Mean ± SD, n=3, (***p<0.0001)



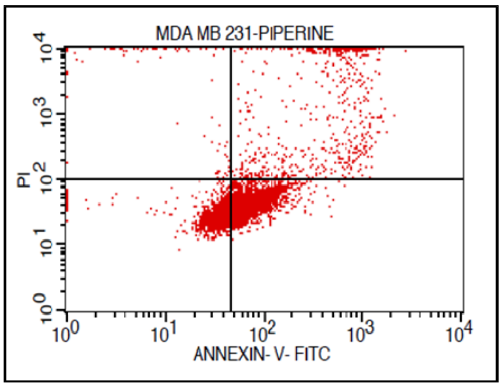


Fig 3: Representative image of flow cytometry analysis of apoptosis in MDA-MB-231 cells after staining with annexin V-FITC/PI: a) vehicle control b) umbelliferone c) piperine d) umbelliferone (IC25) + piperine (IC25)

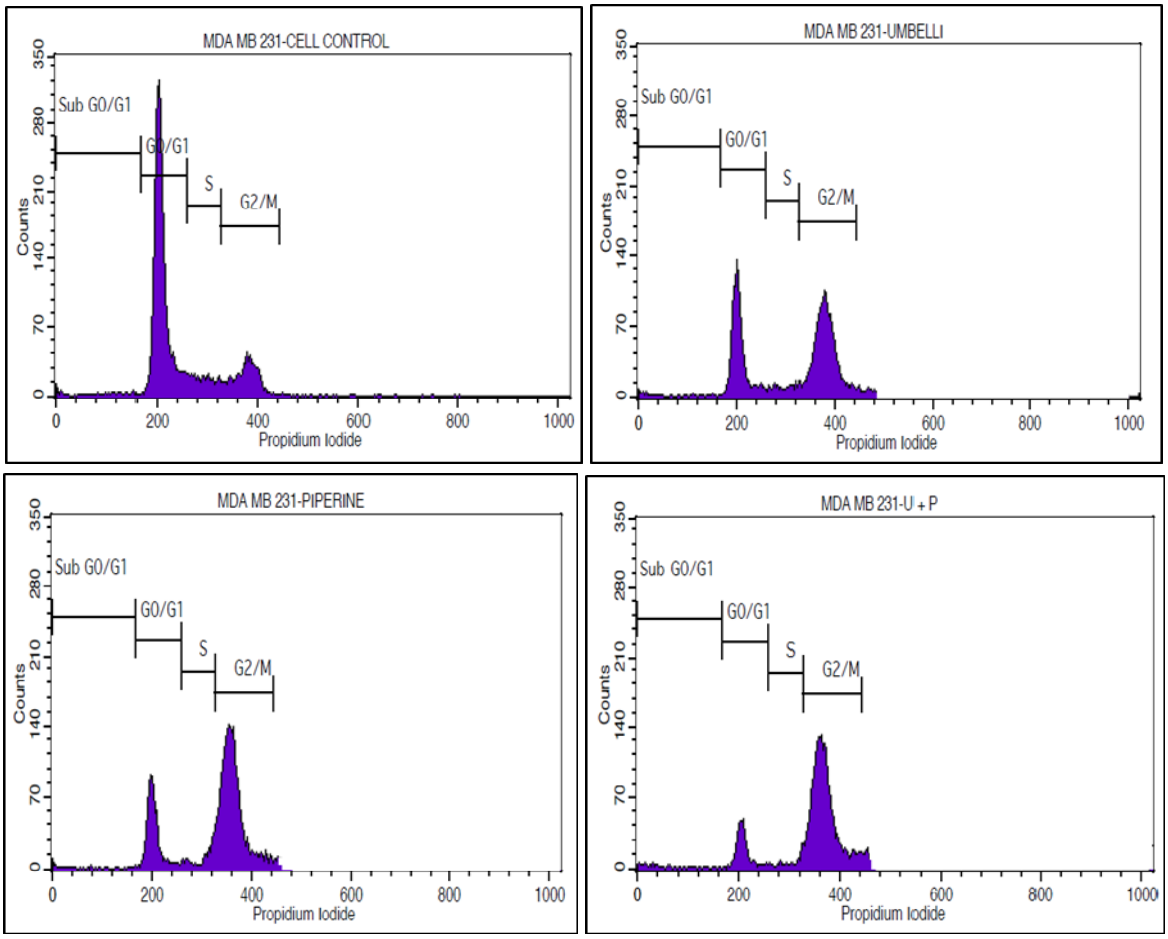


Fig 4: Representative image of cell cycle analysis in MDA-MB-231 cells at 48h incubation by flow cytometry: a) vehicle control b) umbelliferone c) piperine d) umbelliferone (IC_{25}) + piperine (IC_{25})

Table 1: Effect of umbelliferone, piperine and umbelliferone (IC_{25}) + piperine (IC_{25}) on apoptosis and necrosis from MDA-MB-231 cells. one-way ANOVA, followed by *post hoc* Tukey's multiple comparison test. Values are Mean± SD, n=3

Groups	UL	UR	LL	LR
Vehicle Control	0.00±0.00	0.11±0.09[a]	99.63±0.32	0.13±0.12[a]
Umbelliferone	1.45±0.37	9.93±0.86[c]	19.13±0.63	68.50±1.24[b]
Piperine	0.87±0.04	5.44±0.85[b]	27.19±1.40	66.13±1.08[b]
Umbelliferone + Piperine	0.86±0.04	10.60±0.42[d]	14.81±0.94	73.36±1.26[c]

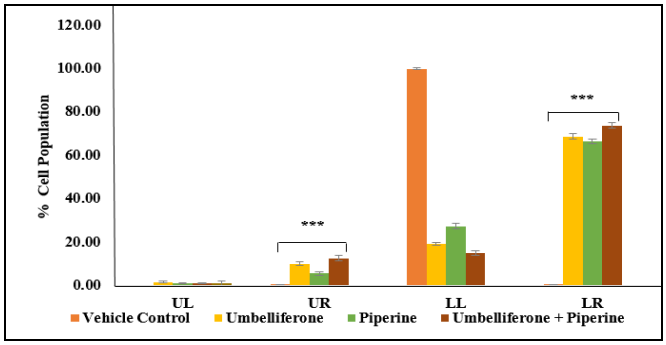
UL: Necrosis UR: End stage of Apoptosis LL: Normal cells LR: Early stage of Apoptosis

Note: Values bearing different superscripts within in a column differ significantly (P<0.0001)

Table 2: Effect of umbelliferone, piperine and umbelliferone (IC_{25}) + piperine (IC_{25}) on cell cycle from MDA-MB-231 cells. One-way ANOVA, followed by *post hoc* Tukey's multiple comparison test. Values are Mean ± SD, n=3

Groups	SubG0/G1	G0/G1	S	G2/M
Vehicle Control	1.90±0.03	72.30±1.16	9.79±0.72	15.68±1.05[a]
Umbelliferone	0.94±0.10	18.62±0.66	5.13±0.73	56.60±1.18[c]
Piperine	1.01±0.15	28.79±0.95	4.50±0.78	43.40±0.83[b]
Umbelliferone + Piperine	1.06±0.22	9.05±0.66	3.31±0.46	58.30±1.11[d]

Note: Values bearing different superscripts within a column differ significantly (P<0.0001)



UL: Necrosis UR: End stage of Apoptosis LL: Normal cells LR: Early stage of Apoptosis

Fig 5: Histogram representation of flow cytometry data for population of cells in early and late stage of apoptosis and necrosis from MDA-MB-231 cells treated with umbelliferone, piperine and umbelliferone (IC_{25}) + piperine (IC_{25}). Values are Mean± SD, n=3, (***p<0.0001)

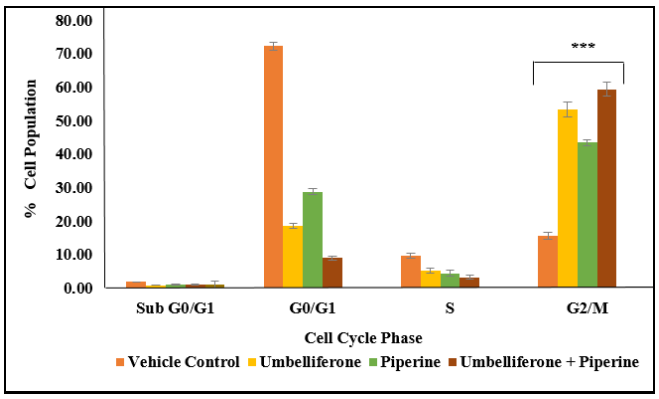


Fig 6: Histogram representation of flow cytometry data for population of cells in Sub G0/G1, G0/G1, S and G2/M phases of cell cycle from MDA-MB-231 cells treated with umbelliferone, piperine and umbelliferone (IC_{25}) + piperine (IC_{25}). Values are Mean ± SD, n=3, (***p<0.0001)

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5. References
1. Seca AML, Pinto DCGA. Plant secondary metabolites as anticancer agents: successes in clinical trials and therapeutic application. International Journal of Molecular Sciences. 2018; 19(1):1-22.
2. Czemplik M, Mierziak J, Szopa J, Kulma A. Flavonoid C-glucosides derived from flax straw extracts reduce human breast cancer cell growth *in vitro* and induce apoptosis. Frontiers in Pharmacology. 2016; 7:282.
3. Wang R, Yang L, Li S, Ye D, Yang L, Liu Q *et al*. Quercetin inhibits breast cancer stem cells *via* down regulation of aldehyde dehydrogenase 1A1 (ALDH1A1), chemokine receptor type 4 (CXCR4), mucin 1 (MUC1) and epithelial cell adhesion molecule (Ep CAM). Medical Science Monitor. 2018; 24:412-420.
4. La porta E, Welsh J. Modelling vitamin D in triple-negative/basal- like breast cancer. Journal of Steroid Biochemistry and Molecular Biology. 2014; 144:65-73.
5. Razak NA, Abu N, Ho WY, Zamberi NR, Tan SW, Alitheen NB *et al*. Cytotoxicity of eupatorin in MCF-7 and MDA-MB-231 human breast cancer cells *via* cell cycle arrest, anti-angiogenesis and induction of apoptosis. Scientific Reports. 2019; 9:1514.
6. Gest C, Joimel U, Huang L, Pritchard LL, Petit A, Dulong C *et al*. Rac3 induces a molecular pathway triggering breast cancer cell aggressiveness: differences in MDA-MB-231 and MCF-7 breast cancer cell lines. BMC Cancer. 2013; 13:63.
7. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nature Reviews Drug Discovery. 2006; 5:219-34.
8. Raguz S, Yague E. Resistance to chemotherapy: new treatments and novel insights into an old problem. British Journal of Cancer. 2008; 99(3):387-391.
9. Sriwiriyajan S, Tedasen A, Lailerd N, Boonyaphiphat P, Nitiruangjarat A, Deng Y *et al*. Anticancer and cancer prevention effects of piperine-free *Piper nigrum* extract on N-nitroso methylurea-induced mammary tumorigenesis in rats. Cancer Prevention Research. 2016; 9(1):74-82.
10. Newman DJ, Cragg GM and Snader KM. Natural products as sources of new drugs over the period 1981-2002. Journal of Natural Products. 2003; 66(7):1022-1037.
11. Cragg GM and Newman DJ. Plants as a source of anti-cancer agents. Journal of Ethno pharmacology. 2005; 100(1-2):72-79.
12. Mazimba O. Umbelliferone: Sources, chemistry and bioactivities review. Bulletin of Faculty of Pharmacy. 2017; 55(2):223-232.
13. Muthu R, Thangavel P, Selvaraj N, Ramalingam R, Vaiyapuri M. Synergistic and individual effects of umbelliferone with 5-flurouracil on the status of lipid peroxidation and antioxidant defense against 1,2-dimethylhydrazine induced rat colon carcinogenesis. Biomedicine and Preventive Nutrition. 2013; 3(1):74-82.
14. Kielbus M, Skalicka-Wozniak K, Grabarska A, Jeleniewicz W, Dmoszynska-Graniczka M, Klatka J *et al*. 7-substituted coumarins inhibit proliferation and migration of laryngeal cancer cells *in vitro*. Anticancer Research. 2013; 33(10):4347-4356.
15. Yu SM, Hu DH, Zhang JJ. Umbelliferone exhibits anticancer activity *via* the induction of apoptosis and cell cycle arrest in HepG2 hepatocellular carcinoma cells. Molecular Medicine Reports. 2015; 12(3):3869-3873.
16. Rather RA, Bhagat M. Cancer chemoprevention and piperine: molecular mechanisms and therapeutic opportunities. Frontiers in Cell and Developmental Biology. 2018; 6:1-12.
17. Philipova I, Valcheva V, Mihaylova R, Mateeva M, Doytchinov I, Stavrakov G. Synthetic piperine amide analogs with ant mycobacterial activity. Chemical Biology and Drug Design. 2018; 91(3):763-768.
18. Soutar DA, Doucette CD, Liwski RS, Hoskin DW. Piperine, a pungent alkaloid from black pepper, inhibits B lymphocyte activation and effector functions. Phitotherapy Research. 2017; 31(3):466-474.
19. Greenshields AL, Doucette CD, Sutton KM, Madera L, Annan H, Yaffe PB. Piperine inhibits the growth and motility of triple-negative breast cancer cells. Cancer Letters. 2015; 357(1):129-140.
20. Zheng J, Zhou Y, Li Y, Xu DP, Li S, Li HB. Spices for prevention and treatment of cancer. Nutrients, 2016; 8(8):495.