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Evaluation of oxidative stress induced cytotoxicity of umbelliferone with or without piperine

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Abstract
Phytocompounds have played an important role as the source of effective anti-cancer agents. Over 60% of the currently used anticancer agents are derived from natural sources including plants, microorganisms and marine organisms. In the present study, oxidative stress indices such as DHE (dihydroethidium) and H_2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate) assays were conducted on MDA-MB-231 cell lines at 48h incubation. The phytocompounds such as umbelliferone and piperine at their respective IC_{50} concentration and a combination of umbelliferone (IC_{25}) with piperine (IC_{25}) showed cytotoxicity due to increased production of reactive oxygen species indicated by an increase in fluorescence under DHE assay and an increase in mean fluorescence intensities by H_2DCFDA assay. Thus, the findings of our study may shed light on oxidative stress-mediated cytotoxicity in triple-negative breast cancer.

Keywords: Umbelliferone, piperine, oxidative stress, DHE, H_2DCFDA

1. Introduction
Medicinal plants have been always an important source for the discovery of new therapeutic agents for diseases [1]. A wide range of phytocompounds present in plants has received considerable attention for the treatment of various diseases. Among various phytochemicals, polyphenols have attracted more attention in the past few years due to their potential health benefits. Among polyphenols, coumarins have recently gained much attention because of their broad pharmacological activities [2]. The umbelliferone (Umb, 7-hydroxycoumarin) is a coumarin derivative, widely distributed in a broad range of plants and exerts various pharmacological effects such as an antidiabetic, antioxidant, ant proliferative and analgesia [3, 4]. Another phyto compound, Piperine (1-Piperoylpiperidine) which is an alkaloid predominantly found in the fruits and roots of *Piper nigrum* L. (black pepper) and *Piper longum* L. (long pepper) species of Piperaceae family [5]. It exhibits anti-inflammatory, anticancer, immunosuppressive, antibacterial, antifungal, antiparasitic, antidiabetic and bio-enhancing properties.

Nowadays, in spite of an increase in pharmacological and clinical advances, cancer is still a major healthcare problem. The imbalanced redox state, cell cycle alterations, increased proliferation and, inflammatory status are the molecular signatures in cancer [6]. The reactive oxygen species (ROS) can play a dual role in all types of cancers. In normal cells, an increase in ROS provokes mitochondrial dysfunction followed by protein oxidation, lipid peroxidation, and DNA damage leading to a pro-oncogenic state [6]. In tumor cells, an increased ROS level can result in cytotoxicity. Some anticancer drugs such as doxorubicin and paclitaxel trigger increased amount of ROS generation in tumor cells resulting in cytotoxicity [7, 8]. Among phytocompounds, polyphenols play a role in both situations. Polyphenols produce chemo-preventive effect due to their antioxidant properties. They counteract ROS production and inhibit oxidative mitochondrial dysfunction and DNA damage [9, 11]. Secondly, polyphenols cause tumor cell death due to their prooxidants properties [12, 13]. The cancer cells contain an increased amount of copper [14], which is an active redox metal when compared to normal cells. The polyphenols catalyze the redox cycle and generate ROS [15, 17] leading to preferential cytotoxicity against cancer cells leaving the normal cells undamaged. Hence, in the present study, we investigated the cytotoxicity potential pertaining to the pro-oxidant property of

Umbelliferone with or without piperine co-exposure in triple-negative breast cancer cell lines.

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. DHE (Thermo Fisher Scientific, #D11347), H_2DCFDA (Thermo Fisher Scientific, #D399), 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, trypsin, streptomycin, penicillin, amphotericin, fetal bovine serum (Hi Media Labs, Mumbai, India) and other reagents of analytical grade were also used in the study.

2.2 Measurement of reactive oxygen species

The IC_{50} of umbelliferone and piperine on MDA-MB-231 cells was found to be 10.31 and 14.28 µM, respectively [18] and the same was considered for the measurement of reactive oxygen species in the present study.

2.2.1. DHE (dihydroethidium) staining using a fluorescent microscope

A cell-permeable fluorescent marker DHE (dihydroethidium, hydroethidine), which upon oxidation by ROS yields the red-fluorescent product 2-hydroxyethidium was used to measure the intracellular generation of ROS (superoxide) [19]. The MDA-MB-231 cells were grown on glass cover-slips in six-well plates. The umbelliferone and piperine at their respective IC_{50} values and a combination of umbelliferone (IC_{25}) and piperine (IC_{25}) were added and incubated for 48h. Later, cells were stained with 20 µM DHE for 30 min and observed under a fluorescent microscope (Lion heart FX, Bio Tek, USA).

2.2.2 H_2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate) staining using flow cytometry

A cell-permeable fluorescent marker H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate, dichlorofluorescin cin diacetate), which upon oxidation by ROS yields 2', 7'-dichlorofluorescin [19] was used to measure the intracellular generation of hydroxyl, peroxyl and other ROS [20] was measured using. The MDA-MB-231 cells were cultured in 6-well plates for 24h at 37°C in a 5% CO_2 humidified atmosphere and were later exposed to umbelliferone and piperine at their respective IC_{50} values and a combination of umbelliferone (IC_{25}) and piperine (IC_{25}) for 48h. Later, the cells were harvested, stained with 10µM H_2DCFDA for 30 min at 37 ^{0}C, in dark and analyzed by flow cytometer (BD Biosciences FACS Calibur, USA) using Fluorescence channel-1 (FL1). The staining levels were measured as mean fluorescence intensities (MFI) using BD Cell Quest™ Pro software, Version 6.

2.2.3 Statistical analysis

The reactive oxygen species as mean fluorescence intensities of 2', 7'- dichlorofluorescin 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 one-way ANOVA followed by *post hoc* Tukey's multiple comparison test was done. All the values were expressed as Mean± SD (Graph Pad Prism, version 5).

3. Results and Discussion

3.1. Measurement of reactive oxygen species

The MDA-MB-231 cells upon treatment with umbelliferone and piperine at their respective IC_{50} concentration and a combination of umbelliferone (IC_{25}) with piperine (IC_{25}) showed increased production of superoxide reactive oxygen species which was indicated in terms of increase in fluorescence (Fig. 1b, 1c, and 1d, respectively) when compared to those observed with vehicle control (1%DMSO) (Fig. 1a) in DHE assay. The H_2DCFDA assay revealed that the cytotoxicity potential in terms of increase in hydroxyl, peroxyl and other reactive oxygen species was significantly (p<0.0001) increased (Fig. 2, 3 and Table 1) in MDA-MB-231 cells treated with umbelliferone and piperine at their respective IC_{50} concentration and a combination of umbelliferone with piperine at their respective IC_{25} concentration.

In conclusion, the results clearly demonstrated that MDA-MB-231 cells upon treatment with test compounds exhibited oxidative stress *i.e* pro-oxidant status in the order of umbelliferone+piperine > umbelliferone > piperine. The cytotoxicity can be attributed to the reactive oxygen species which triggers the depolarization of mitochondrial membrane potential (MMP), leading to the release of cytochrome c, activation of caspases and induction of apoptosis [6]. Further, the pro-oxidant activity of piperine [21] might have additionally contributed to the enhanced cytotoxicity of the combination. Thus, the findings of our study may shed light on the application of umbelliferone alone or in combination with piperine for the treatment of triple-negative breast cancer.

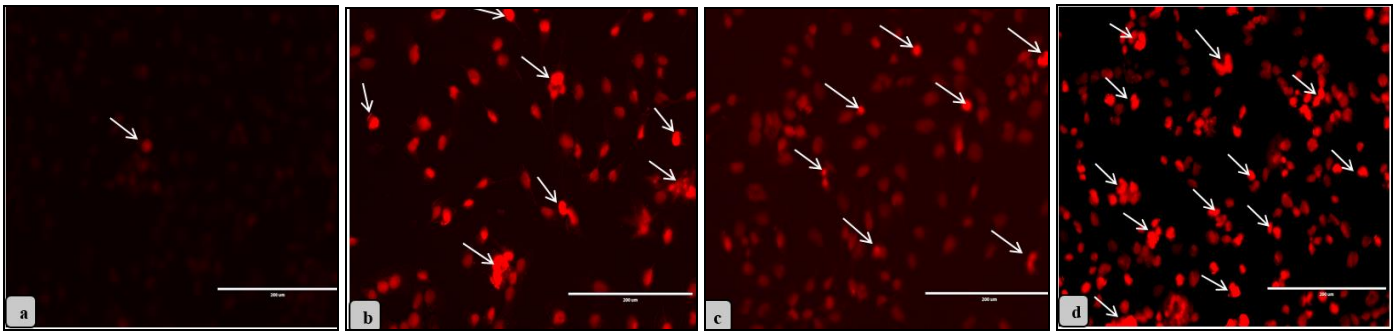

Fig 1: Representative image of fluorescence microscopy analysis of reactive oxygen species in MDA-MB-231 cells after staining with DHE: a) vehicle control b) umbelliferone c) piperine d) umbelliferone (IC25) + piperine (IC25)a

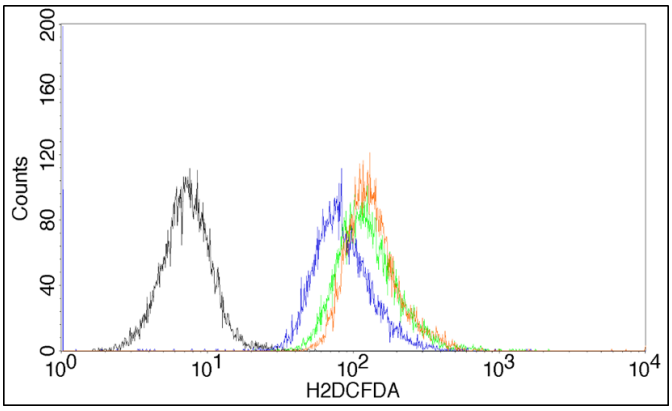

Fig 2: Representative Overlay of flow cytometry analysis of reactive oxygen species of vehicle control, umbelliferone, piperine and umbelliferone (IC_{25}) + piperine (IC_{25}) in MDA-MB-231 cells after staining with H_2DCFDA

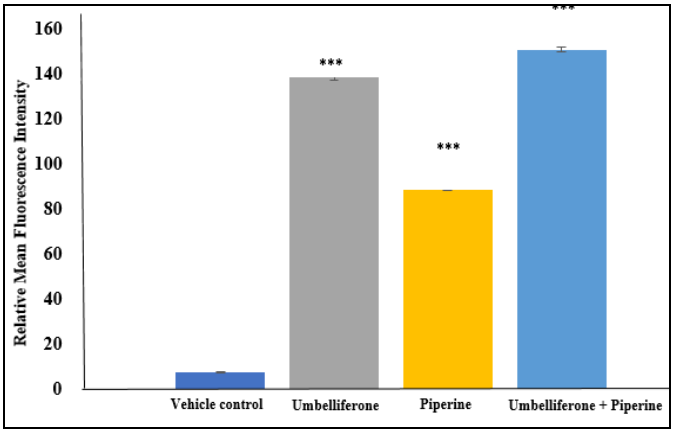

Fig 3: Bar graph representation of flow cytometry data for relative mean florescence intensity of H_2DCFDA staining from MDA-MB-231 cells treated with umbelliferone, piperine, and umbelliferone (IC_{25}) + piperine (IC_{25}). Values are Mean ± SD, n=3, (***p<0.0001)

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

Groups	Relative mean Fluorescence intensity (MFI)
Vehicle Control	7.62 ± 0.36^{a}
Umbelliferone	138.52 ± 1.26^{c}
Piperine	88.33 ± 0.92^{b}
Umbelliferone + Piperine	150.60 ± 1.04^{d}

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

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References
1. Hosseini A, Ghorbani A. Cancer therapy with phytochemicals: Evidence from clinical studies. Avicenna Journal of Phytomedicine. 2015; 5(2):84-97.
2. Mirunalini S, Krishnaveni M. Coumarin: A plant derived polyphenol with wide biomedical applications. International Journal of Pharm Tech Research. 2011; 3(3):1693-1696.
3. Ramesh B, Viswanathan P, Pugalendi KV. Protective effect of umbelliferone on membranous fatty acid composition in streptozotocin-induced diabetic rats. European Journal of Pharmacology. 2007; 566(1-3):231-239.
4. Lacatusu I, Badea N, Murariu A, Oprea O, Bojin D, Meghea A. Antioxidant activity of solid lipid nanoparticles loaded with umbelliferone. Soft Matter. 2012; 11:75-84.
5. Zheng J, Zhou Y, Li Y, Xu DP, Li S, Li HB *et al*. Spices for prevention and treatment of cancers. Nutrients. 2016; 8(8):1-35.
6. Losada-Echeberria M, Herranz-Lopez M, Micol V, Barrajon-Catalan E. Polyphenols as promising drugs against main breast cancer signatures. Antioxidants. 2017; 6(4):1-24.
7. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nature Reviews Drug Discovery. 2013; 12:931-947.
8. Hecht F, Pessoa CF, Gentile LB, Rosenthal D, Carvalho DP and Fortunato RS. The role of oxidative stress on breast cancer development and therapy. Tumour Biology. 2016; 37(4):4281-4291.
9. Khan N, Afaq F, Mukhtar H. Cancer chemoprevention through dietary antioxidants: progress and promise. Antioxidants and Redox Signaling. 2008; 10(3):475-510.
10. Stoner GD and Mukhtar H. Polyphenols as cancer chemo preventive agents. Journal of Cellular Biochemistry. Suppl. 1995; 22:169-180.
11. Mileo AM, Miccadei S. Polyphenols as modulator of oxidative stress in cancer disease: New therapeutic strategies. Oxidative Medicine and Cellular Longevity. 2016; 17:1-18.
12. Elbling L, Weiss RM, Teufelhofer O, Uhl M, Knasmueller S, Schulte-Hermann R *et al*. Green tea extract and (-)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. FASEB Journal. 2005; 19(7):807-809.
13. Hadi SM, Bhat SH, Azmi AS, Hanif S, Shamim U, Ullah MF. Oxidative breakage of cellular DNA by plant polyphenols: A putative mechanism for anticancer properties. Semin. Cancer Biology. 2007; 17(5):370-376.
14. Denoyer D, Masaldan S, La Fontaine S, Cater MA. Targeting copper in cancer therapy: 'copper that cancer'. Metallomics. 2015; 7(11):1459-1476.
15. Decker EA. Phenolics: Prooxidants or antioxidants? Nutrition Reviews. 1997; 55(11):396-398.
16. Li Y, Trush MA. Reactive oxygen-dependent DNA damage resulting from the oxidation of phenolic compounds by a copper-redox cycle mechanism. Cancer Research. 1994; 54(7):1895s-1898s.
17. Khan HY, Zubair H, Faisal M, Ullah MF, Farhan M, Sarkar FH *et al*. Plant polyphenol induced cell death in human cancer cells involves mobilization of intracellular copper ions and reactive oxygen species generation: A mechanism for cancer chemo preventive action. Molecular Nutrition and Food Research. 2014; 58:437-446.
18. Rashmi R, Prakash N, Rathnamma D, Narayana swamy HD, Narayana Swamy M, Santhosh CR *et al*. Evaluation of Oxidative Stress Induced Cytotoxicity of umbelliferone with or with piperine on breast cancer cell lines. Journal of Entomology and Zoology Studies, 2019;
19. Owusu-Ansah E, Yavari A, Banerjee U. A protocol for *in vivo* detection of reactive oxygen species. Protocol Exchange, 2008. Doi: 10.1038/Nprot200823.
20. Halliwell B, Whiteman M. Measuring reactive species

and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean. British Journal of Pharmacology. 2004; 142(2):231-255.
21. Rather RA, Bhagat M. Cancer chemoprevention and piperine: molecular mechanisms and therapeutic opportunities. Frontiers in Cell and Developmental Biology. 2018; 6:1-12.