Evaluation of oxidative stress induced cytotoxicity of umbelliferone with or without piperine on triple-negative breast cancer


Abstract
Phytochemicals 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, marine organisms, and microorganisms. In the present study, oxidative stress indices such as DHE (dihydroethidium) and H$_2$DCFDA (2', 7'-dichlorodihydrofluorescein diacetate) assays were conducted on MDA-MB-231 cell lines at 48h incubation. Phytochemicals such as umbelliferone and piperine at their respective IC$_{50}$ concentration and 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$_2$DCFDA 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$_2$DCFDA

1. Introduction
Medicinal plants have been always an important source for the discovery of new therapeutic agents for the diseases (Hosseini and Ghorbani, 2015) [1]. A wide range of phytochemicals present in plants has received considerable attention for the treatment of various diseases. Among various phytochemicals, polyphenols have attracted considerable interest in the past few years due to their potential health benefits. Among polyphenols, coumarins have recently attracted much attention because of their broad pharmacological activities (Mirunalini and Krishnaveni, 2011) [2]. Umbelliferone (Umb, 7-hydroxycoumarin) is a coumarin derivative, widely distributed in a broad range of plants and exerts various pharmacological effects such as an anti-inflammatory, antioxidant, antiplatelet, and analgesic (Ramesh et al., 2007; Lacatusu et al., 2013) [3, 4]. Another phytochemical, 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 (Zheng et al., 2016) [5]. It exhibits anti-inflammatory, anticancer, immunosuppressive, antibacterial, antifungal, antiparasitic, antidiabetic and bio-enhancing properties.

Nowadays, in an increase of pharmacological and clinical advances, breast cancer is still a major healthcare problem, causing morbidity and mortality in women worldwide. Likewise, other neoplasms breast cancer shares some molecular signatures such as an imbalanced redox state, cell cycle alterations, increased proliferation and inflammatory status. Its therapy is of great challenge in modern medicine to conquer its morbidity and mortality (Losada-Echeberria et al., 2017) [6].

Reactive oxygen species can play a dual role (Gorrini et al., 2013) [7], not only in breast cancer (Hecht et al., 2016) [8] but also in all other 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. On the other hand, once tumor cells have developed and increased ROS generation can lead to tumor cell death. This fact has been linked to some anticancer drugs such as doxorubicin and paclitaxel.
Among phytocompounds, polyphenols play a role in both situations. As polyphenols are antioxidants, they counteract ROS production and inhibit oxidative DNA damage and mitochondrial dysfunction thereby acts as chemopreventive agents (Khan et al., 2008; Stoner and Mukhtar, 1995; Mileo and Miccadi, 2016) [9–11]. Secondly, polyphenols can also act as prooxidants, leading to tumor cell death (Eibling et al., 2005; Hadi et al., 2007) [12, 13]. Cancer cells contain an increased amount of copper (Denoyer et al., 2015) [14], which is an active redox metal when compared to normal cells. Polyphenols catalyze the redox cycle and generate ROS formation (Decker, 1997; Li and Trush, 1994; Khan et al., 2014) [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), Dimethy sulfoxide (Sigma, #D8418) were purchased from Sigma-Aldrich Chemical Co. Ltd. DHE (ThermoFisher Scientific, #D11347), H2DCFDA (ThermoFisher 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 (HiMedia Labs, Mumbai, India) and other reagents of analytical grade were also used in the study.

2.2 Measurement of reactive oxygen species

The IC50 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

The intracellular generation of ROS (superoxide) was measured using a cell-permeable fluorescent marker DHE (dihydroethidium, hydroethidine), which upon oxidation by ROS yields the red-fluorescent product 2,7’-dichlorodihydrofluorescein diacetate (Owusu-Ansah et al., 2008) [19]. MDA-MB-231 cells were cultured in 6-well plates for 24h at 37°C in a 5% CO2 humidified atmosphere and were later exposed to umbelliferone and piperine at their respective IC50 values and a combination of umbelliferone (IC25) and piperine (IC25) for 48h. Later, the cells were harvested, stained with 10 μM H2DCFDA for 30 min at 37°C, in dark and analyzed by flow cytometer (BD Biosciences FACSCalibur, USA) using Fluorescence channel-1 (FL1). Staining levels were measured as mean fluorescence intensities (MFI) using BD CellQuest™ Pro software, Version 6.

2.3 Statistical Analysis

Reactive oxygen species as mean fluorescence intensities of 2’, 7’-dichlorofluorescin were analyzed by using BD CellQuest™ 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 FACSCalibur, USA). 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

MDA-MB-231 cells upon treatment with umbelliferone and piperine at their respective IC50 concentration and combination of umbelliferone (IC25) with piperine (IC25) 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. H2DCFDA assay revealed that the cytotoxicity potential in terms of increase in hydroxyl, peroxy, 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 IC50 concentration and combination of umbelliferone with piperine at their respective IC25 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 (Osada-Echeberria et al., 2017) [6]. Further, the pro-oxidant activity of piperine (Rather and Bhagat, 2018) [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 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 (IC<sub>25</sub>) + piperine (IC<sub>25</sub>)

Fig 2: Representative Overlay of flow cytometry analysis of reactive oxygen species of vehicle control, umbelliferone, piperine and umbelliferone (IC<sub>25</sub>) + piperine(IC<sub>25</sub>) in MDA-MB-231 cells after staining with H<sub>2</sub>DCFDA
Table 1: Effect of umbelliferone, piperine and umbelliferone (IC_{25}) + piperine(IC_{25}) on reactive oxygen species at 48h incubation from MDA-MB-231 cells. One-way ANOVA, followed by post hoc Tukey’s multiple comparison test. Values are Mean ± SD, n=3

<table>
<thead>
<tr>
<th>Groups</th>
<th>Relative mean Fluorescence intensity (MFI)</th>
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</thead>
<tbody>
<tr>
<td>Vehicle Control</td>
<td>7.62 ± 0.36(^a)</td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>138.52 ± 1.26(^c)</td>
</tr>
<tr>
<td>Piperine</td>
<td>88.33 ±0.92(^b)</td>
</tr>
<tr>
<td>Umbelliferone + Piperine</td>
<td>150.60 ±1.04(^d)</td>
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Note: Values bearing different superscripts within a column differ significantly (\(P<0.0001\))
damage resulting from the oxidation of phenolic compounds by a copper-redox cycle mechanism. Cancer Res. 1994; 54(7):1895s-1898s.


