Cytotoxicity screening of esculin with or without piperine on breast cancer cell lines


Abstract
Recently, phytochemicals have gained considerable attention of the scientific community for their effective role in cancer therapy. In the present study, we investigated the cytotoxicity potential of esculin with or without piperine on breast cancer cell lines viz., MCF-7 and MDA-MB-231. MTT [3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide] assay was conducted at 48h incubation to determine percent cell viability. IC₅₀ values of esculin and piperine for MCF-7 were 20.35 and 17.83μM, respectively. Likewise, IC₅₀ values of esculin and piperine for MDA-MB-231 were 22.65 and 14.28μM, respectively. A combination of esculin (IC₅₀) and piperine (IC₅₀) on MCF-7 and MDA-MB-231 showed percent cell viability of 37.90±0.03 and 10.12±0.03, respectively. This study suggests that the cytotoxicity of esculin and piperine is dose-dependent. The in vitro cytotoxicity potential of esculin and piperine alone and in their combination in terms of percent cell viability proved to be more effective on MDA-MB-231 cells.

Keywords: esculin, piperine, cytotoxicity, MCF-7, MDA-MB-231

1. Introduction
Breast cancer, a global menace is one of the most common malignancies and the second leading cause of death among women worldwide [1, 2]. In 2018, an estimated 6,27,000 women died from breast cancer which is approximately 15% of all cancer deaths among women. Breast cancer incidence rates are higher among women in more developed countries and also rates are increasing in nearly every region globally [3]. Several factors such as gender, diet, family history, lifestyle, endocrine dysfunction are associated with breast cancer [4]. Its most common classification is based on hormone and growth factor receptor expression. Breast cancer cells that have gained interest amongst researchers are the oestrogen receptor (ER) positive breast cancer, MCF-7 [5] and oestrogen receptor (ER) negative breast cancer, MDA-MB-231 [6]. One of the major challenges for the treatment of breast cancer is its heterogeneous nature, which determines the therapeutic options [7, 8]. Phytochemicals or secondary metabolites are non-nutritious, non-toxic and have a wide range of biological activities viz., anti-inflammatory, anti-proliferative, antioxidant and anticancer [9]. They possess anticancer activity by acting against oxidative stress, DNA damage or by blocking several cancer pathways [10, 11]. Coumarins are widely distributed in plants and have recently attracted much attention because of their broad biological activities [12, 13]. Esculin (6, 7-dihydroxycoumarin-6-o-glucoside) is a coumarin derivative having antioxidant [12], anti-inflammatory [14], gastro-protectant [15], anti-diabetic, analgesic, antibacterial and anticoagulant effect [16, 17]. Piperine (1-Piperoylpiperidine) 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 [18]. It possesses antioxidant, anti-inflammatory, immunomodulatory, anti-asthmatic, anti-convulsant, anti-mutagenic, anti-mycobacterial, anti-amoebic and anticancer activities [19, 20]. The cytotoxicity of esculin and also its combinational cytotoxicity with piperine has not been reported in breast cancer cell lines. Hence, the present investigation was undertaken to determine the cytotoxicity of esculin with or without piperine on breast cancer cell lines.

2. Materials and Methods
2.1 Materials
Esculin (Sigma, #E8250), Piperine (Sigma, #P49007), Dimethly sulfoxide (Sigma, #D8418)
were purchased from Sigma-Aldrich Chemical Co. Ltd. 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, MTT [3-(4,5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide], trypsin, streptomycin, penicillin, amphotericin, fetal bovine serum, absolute ethanol (Hi-Media Labs, Mumbai, India) and other reagents of analytical grade were also used in the study.

2.2 Cell lines and culture conditions
Breast cancer cell lines such as MCF-7 and MDA-MB-231 (National Centre for Cell Science, Pune, India) were used for in vitro cytotoxicity assay. Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (4.5g/L Glucose), supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (4mM), penicillin 10,000 units/mL, streptomycin 1000μg/mL and Amphotericin-B solution 25μg/mL. Cells were incubated at 37°C in a humidified atmosphere of 95 percent air and 5 percent CO2. Following 24h of incubation, the adherent cells were detached using trypsin-EDTA solution 1X/0.25%. 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
MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was carried out to determine the cytotoxicity of esculin, piperine and combination of esculin with piperine on MCF-7 and MDA-MB-231 cell lines. Cell suspension of 200μL was seeded in 96-well microplates (Corning®, USA) at a density of 20,000 cells/well and incubated in 5 percent CO2 incubator at 37 °C for 24h, after which the cells were exposed to a graded concentration of 3.125, 6.25, 12.5, 25 and 50 and 100μM of esculin, piperine and incubated 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. This was followed by the removal of MTT solution and then the addition of 100μL of DMSO to dissolve insoluble formazan crystals formed within mitochondria of viable cells. The plate was incubated with DMSO for 5min with gentle shaking. Cell viability was determined by measuring the absorbance on a microplate reader (ELx800, Bio Tek, USA) at 630nm. The concentration of esculin and piperine that resulted in 50 percent inhibition of cell growth was calculated as the half-maximal inhibitory concentration (IC50) by constructing a dose-response curve. The IC50 for each cell line was determined by a linear curve fit generated after obtaining dose-response to five different concentrations of esculin and piperine. Cell viability percentages (y-axis) were plotted against increasing concentrations of esculin and piperine on the x-axis. IC50 values were estimated by using the linear regression equation y=mx+c; where 50 is substituted for y, yielding x as the IC50 value.

Later, the cell viability percent for the combination of esculin (IC25) and piperine (IC25) on MCF-7 and MDA-MB-231 cell lines was carried out at 48h incubation.

2.4 Statistical Analysis
The data of in vitro cytotoxicity studies were subjected to linear regression analysis to obtain dose-response curves and IC50 values. The student t test was carried out and all the values were expressed as Mean±SD (GraphPad Prism, Version 5).

3. Results and Discussion
3.1 In Vitro Cytotoxicity Assay
The cytotoxicity of esculin and piperine on both MCF-7 and MDA-MB-231 cell lines was dose-dependent (Fig.1a and 3b, respectively). IC50 values of esculin and piperine for MCF-7 were 20.35 and 17.83μM, respectively (Fig.1b and 1c, respectively). Likewise, IC50 values of esculin and piperine for MDA-MB-231 were 22.65 and 14.28μM, respectively (Fig.2b and 2c, respectively). A combination of esculin (IC25) with piperine (IC25) on MCF-7 and MDA-MB-231 showed a percent cell viability of 37.90±0.03 and 10.12±0.03, respectively (Fig.1d and 4a; 2d and 4b, respectively). The cytotoxicity of esculin and piperine in terms of percent cell viability on both MCF-7 and MDA-MB-231 cell lines was dose-dependent. Based on IC50 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 esculin and piperine. Further, a combination of esculin with piperine produced enhanced cytotoxicity when compared to those observed with esculin or piperine alone treated group. The variation in the cytotoxicity of test compounds towards each breast cancer cell lines was different which in turn depends on the classification and degree of malignancy of cancer cells [21]. In conclusion, triple-negative breast cancer is susceptible to esculin, piperine, and their combination. The successful results of the present study and also an additional set of investigations could help to understand the possibility of a combination of the phytocompounds with the available conventional drug to encounter drug resistance and adverse effects.

![Fig 1: Representative images of cytotoxic effect of a) vehicle control b) esculin c) piperine d) combination [esculin (IC25) + piperine (IC25)] on MCF-7 cell lines at 48h incubation (10X)](image)
**Fig 2:** Representative images of cytotoxic effect of a) vehicle control b) esculin c) piperine d) combination [esculin (IC$_{25}$) + piperine (IC$_{25}$)] on MDA-MB-231 cell lines at 48h incubation (10X)

**Fig 3:** Histogram depiction of dose dependent cytotoxicity data of esculin 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)
**Conflict of interests**
The authors declare that they have no conflict of interest.

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**References**