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Cytotoxicity screening of esculin with or without piperine

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Abstract
Recently, phytocompounds 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. The MTT [3-(4, 5-dimethylthiazolyl- 2)-2, 5- diphenyltetrazolium bromide] assay was conducted at 48h incubation to determine percent cell viability. The IC_{50} values of esculin and piperine for MCF-7 were 20.35 and 17.83µM, respectively. Likewise, IC_{50} values of esculin and piperine for MDA-MB-231 were 22.65 and 14.28µM, respectively. A combination of esculin (IC_{25}) and piperine (IC_{25}) 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
Phytochemicals or secondary metabolites are non-nutritive, non-toxic and have a wide range of biological activities *viz.*, anti-inflammatory, anti-proliferative, antioxidant and anticancer [1, 2]. They produce anticancer activity by acting against oxidative stress, DNA damage or by blocking several cancer pathways [3, 4]. The coumarins are widely distributed in plants and have recently attracted much attention because of their broad biological activities [5, 6]. The esculin (6, 7-dihydroxycoumarin-6-o-glucoside) is a coumarin derivative having antioxidant [5], anti-inflammatory [7], gastro-protectant [8], antidiabetic, analgesic, antibacterial and anticoagulant effect [9, 10]. The 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 [11]. It possesses antioxidant, anti-inflammatory, immunomodulatory, anti-asthmatic, anti-convulsant, anti-mutagenic, anti-mycobacterial, anti-amoebic and anticancer activities [12, 13]. The cytotoxicity of esculin and also its combinatorial cytotoxicity with piperine has not been reported on breast cancer cell lines. Hence, the present investigation was undertaken to determine the cytotoxicity of esculin with or without piperine.

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)-2, 5-diphenyltetrazolium bromide], fetal bovine serum, trypsin, penicillin, streptomycin, amphotericin, 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. The 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), streptomycin 1000µg/mL, penicillin 10,000 units/mL and Amphotericin-B solution 25µg/mL. The cells were incubated at 37°C in a humidified atmosphere of 95 percent air and 5 percent 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 Bio systems, USA) based on the trypan blue dye exclusion method.

2.3 Determination of cytotoxicity

The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-iphenyltetrazolium 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. The 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 of 3.125, 6.25, 12.5, 25, 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 3h 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. The cell viability was determined by measuring the absorbance on a microplate reader (ELx800, Bio Tek, USA) at λmax 570 nm. The concentration of esculin and piperine that resulted in 50 percent 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 esculin and piperine. The cell viability percentages (y-axis) were plotted against increasing concentrations of esculin and piperine on the x-axis. The IC_{50} values were estimated by using the linear regression equation

$$y = mx + c$$

Where 50 is substituted for y, yielding x as the IC_{50} value

$$\text{Cell Viability (\%)} = \frac{\text{Number of cells treated with the test item}}{\text{Number of untreated cells}} \text{ X } 100$$

Later, the cell viability percent for the combination of esculin (IC_{25}) and piperine (IC_{25}) 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 IC_{50} values. The student t-test was carried out and 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 esculin and piperine on both MCF-7 and MDA-MB-231 cell lines was dose-dependent (Fig.3a and 3b, respectively). The IC_{50} values of esculin and piperine for MCF-7 were 20.35 and 17.83µM, respectively (Fig.1b and 1c, respectively). Likewise, IC_{50} 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 (IC_{25}) with piperine (IC_{25}) 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 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 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. In the present study, the variation in the cytotoxicity of test compounds towards each breast cancer cell lines was different which depends on the classification and degree of malignancy of cancer cells [14, 15]. 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.

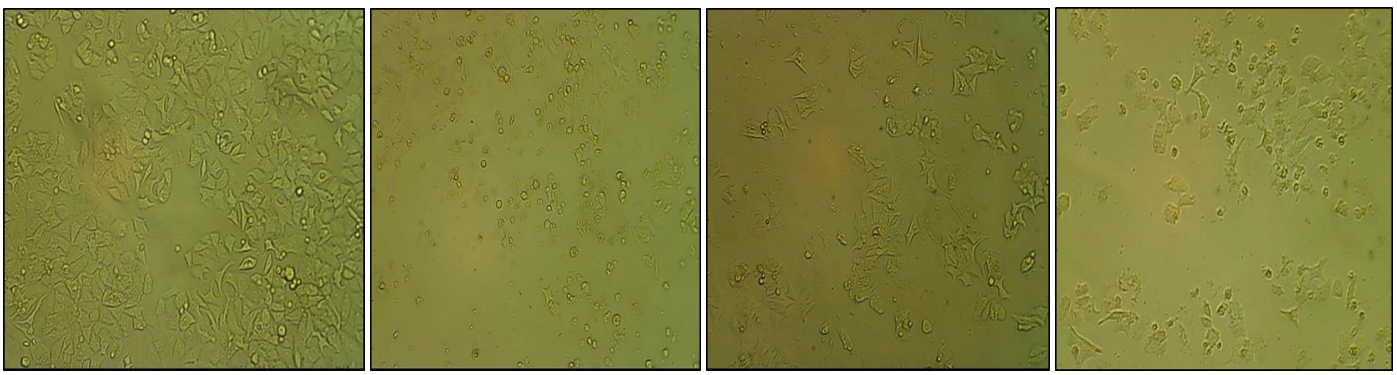

Fig 1: Representative images of cytotoxic effect of a) vehicle control b) esculin c) piperine d) combination [esculin (IC_{25}) + piperine (IC_{25})] on MCF-7 cell lines at 48h incubation (10X)

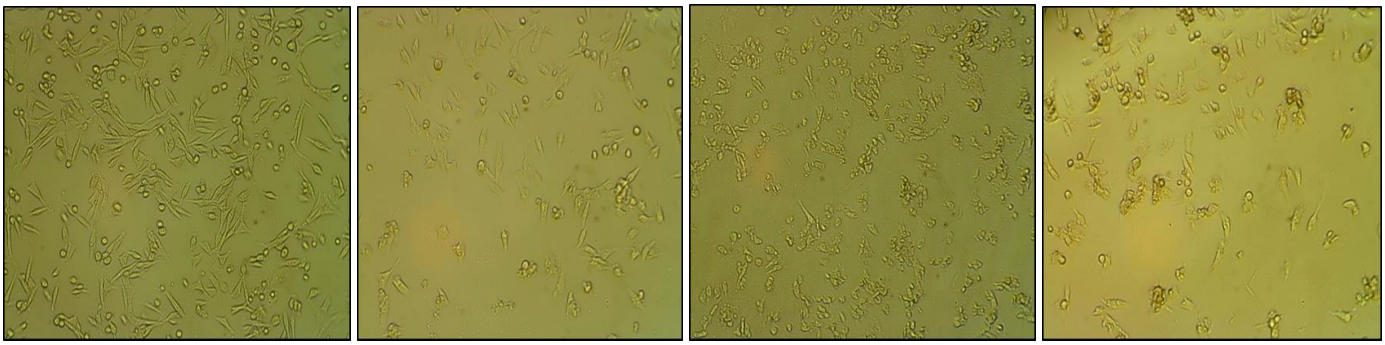

Fig 2: Representative images of cytotoxic effect of a) vehicle control b) esculin c) piperine d) combination [esculin (IC_{25}) + piperine (IC_{25})] on MDA-MB-231 cell lines at 48h incubation (10X)

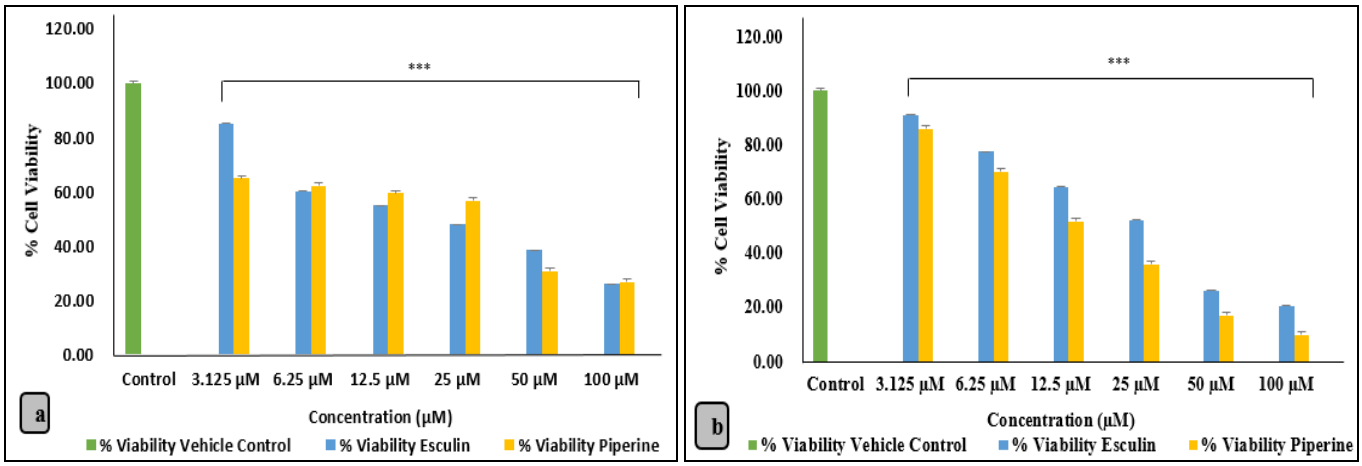

Fig 3: Histogram depiction of dose dependent cytotoxicity data of esculin and piperine as determined by MTT assay on (a) MCF-7 and (b) MDA-MB-231 cell lines. Values are Mean ± SD, n=3q, (***p<0.0001)

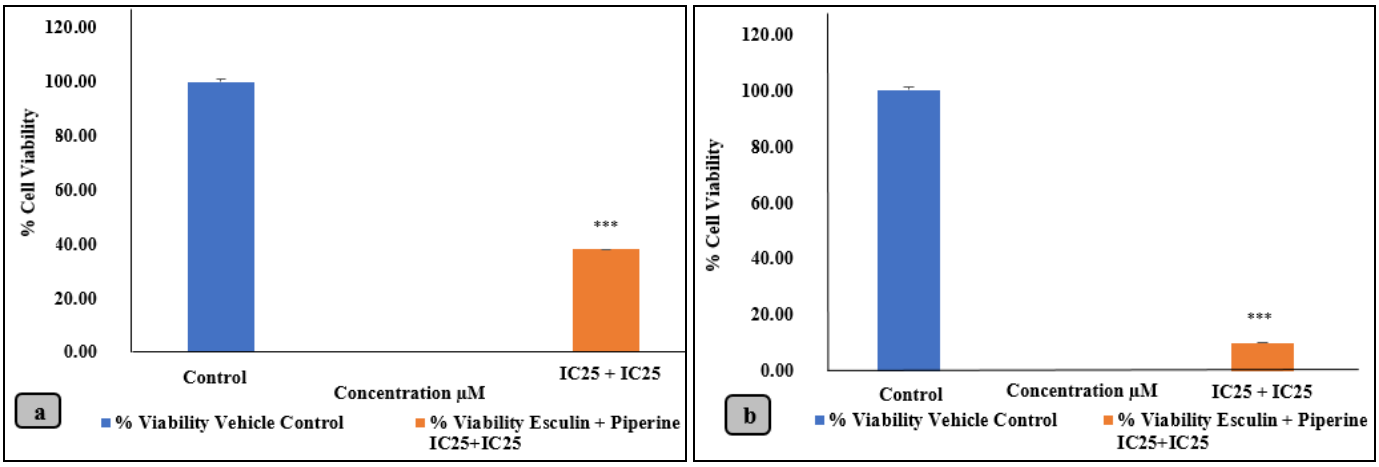

Fig 4: Bar graph depiction of cytotoxicity data of combination studies [(esculin (IC25) + piperine (IC25)] as determined by MTT assay on (a) MCF-7 and (b) MDA-MB-231 cell lines. Values are Mean± SD, n=3, (***p<0.0001)

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