INTRODUCTION

Cancer is one of the major health problems worldwide, and according to cancer statistics 2015, it is estimated that one in eight deaths are due to cancer and this figure is expected to exceed from 2012 to 2023 [1, 2]. Cancer, by and large, is an environmentally determined disease with diets playing a major role. Dietary patterns, foods, nutrients and other dietary constituents are associated with the risk for several types of cancer. It has been estimated that 35% of cancer deaths may be related to dietary factors [3]. Currently, cytotoxic chemotherapy, either alone or in combination with surgery and radiotherapy are the conventional approaches to treat cancer. However, it has been reported that the conventional methods of treatment are often accompanied with many complications such as endometriosis, blood clots, vomiting, and hair loss [4]. In the majority of the cases, cisplatin and doxorubicin are used as chemotherapeutic agents in the treatment of cancer. Both cisplatin (cis-diammine di-chromatoplatinum (II), CDDP) and doxorubicin are highly effective in treating several types of cancers, including ovarian, bladder, lung, cervical, testicular and breast cancers [5-7]. Based on the antitumor activity of cisplatin and doxorubicin on several cancer cell lines, combinational chemotherapy approach is being used to treat cancer. However, treatment with chemotherapeutic drugs alone or in combination can cause several side effects, including nephrotoxicity, peripheral neuropathy, ototoxicity and cardiotoxicity. Therefore, a new combination treatment with increased efficacy and low toxicity is necessary [8-9]. In this connection, recent research focuses on identification of a wide range of phytochemicals with chemopreventive activity against cancer. Many epidemiological pieces of evidence indicate that the frequent consumption of fruit and vegetables reduces the risk of a number of cancers [10, 11]. The combination of plant phytochemicals with conventional chemotherapeutic agents could be a new approach to enhance efficacy while reducing toxicity to normal tissues. Recently, flavonoids received much attention for their health benefits, including anticancer properties possessing antioxidant properties [12-14]. Flavonoids are widespread in various natural sources including apples, tea, broccoli onions, leeks, citrus fruits and grapes, red wines and gingko biloba and is considered to have anti-cancer potential and exerts cytotoxic effects in many types of cancer cells [15]. The majority of the studies revealed the importance of kaempferol as a very promising anticancer drug candidate as it is proved to play a key role in many cellular signal transduction pathways like apoptosis, angiogenesis, inflammation and metastasis. It is also reported that kaempferol inhibits cancer cell growth, simultaneously preserves normal cell viability. In some cases, it is also proved to exert protective effect [10, 17].

The present study reports the results of combination studies using kaempferol with cisplatin and doxorubicin. The biological activities of the kaempferol, doxorubicin and cisplatin alone and in combination have indicated the ability of kaempferol to synergistically potentiate the antitumor effects of doxorubicin and cisplatin on human colon cancer and breast cancer cell lines (HCT-15 and MDA MB 231).

MATERIALS AND METHODS

Materials

Human colorectal cancer (HCT-15) and Human breast cancer cells (MDA-MB 231) were procured from National centre for cell sciences, Pune (NCFS) and all chemicals used in the present study were...
purchased from Sigma Chemical Co. Kaempferol was obtained from Calbiochem, Merck life sciences Pvt.

Cell culture
The cancer cell lines were maintained using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics penicillin (50 U/ml) and streptomycin sulphate (50µg/ml). The cells were maintained at 37 °C with 5% CO2 in a humidified atmosphere. To maintain sub-confluent state, the cells were sub-cultured twice in a week using 0.1% trypsin with 0.5 mmol EDTA. Master and working banks were maintained under liquid nitrogen temperatures for future experimental studies. All compounds were dissolved in ethanol, not exceeding the concentration of 0.01%.

Morphological analysis
Cells were plated at 2×10^5 cells/well in a 6 well plate under the standard culture conditions as described above. The day after initial seeding, cells were treated with ethanol (0.01% v/v) which serve as a control and also with different doses of kaempferol (5, 10, 25, 50, 100 and 150 µg/ml) or doxorubicin or cisplatin alone (5, 10, 25 and 50 µg/ml) or cisplatin alone (5, 10, 25 and 50 µg/ml). After 24 h of these treatments, morphological alterations in cells were observed with phase contrast microscopy and photomicrographs were taken.

Assessment of growth inhibitory effect
The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay [3] was used to screen anti-proliferative activity of cisplatin, doxorubicin and kaempferol on HCT-15 and MDA MB 231 cancer cells. Briefly, the cells were seeded into 96-well plates at a density of 2.5×10^4 to 3.5×10^4 cells/well separately. After one-day incubation and attachment, the cells were treated with different concentrations of compounds and incubated for 24 h. Following washing with phosphate buffer saline (PBS), the cells were incubated with MTT solution (1 ml) for 4 h. Blue coloured crystals indicate the formation of formazon salts. MTT was removed and followed by addition of 1 ml Dimethyl sulfoxide (DMSO). The absorbance was measured after 1 h using microplate reader (Wallac 1420 Multilabel counter, PerkinElmer) at a wavelength of 560 nm. The data were presented as percent post-treatment recovery (% live cells), whereas the absorbance from untreated control cells was defined as 100% live cells. IC50, that is, the concentration of the compound required to inhibit cell growth by 50%, was determined.

Evaluation of drug interaction
Combination studies were performed on HCT-15 and MDA MB 231 with kaempferol, doxorubicin and cisplatin. The cells were treated with kaempferol below its IC50 concentrations (1/2, 1/4 and 1/6) i.e. 60, 30 and 15 µg/ml on HCT-15; whereas 32, 18 and 9 µg/ml on MDA MB 231 along with anti-cancer drugs doxorubicin and cisplatin (1, 5, 10, 25 and 50 µg/ml) separately. Cell percentage viability was determined after 24 h of incubation by the mentioned MTT assay. In the assessment of synergism, the combination index (CI) method (Chou and Talalay) was used. The CI's were calculated by the Chou-Talalay equation, which takes into account of both the potency (Dx, IC50) and shape of the dose-effect curve. The general equation for the classic isobologram (CI=1) is given by

\[ CI = \frac{[D_1][D_2]}{[D_1][D_2]} + \frac{[D_2][D_1]}{[D_1][D_2]} \]  

Where (D1) and (D2) are the doses (concentrations) of (D1) (drug#1, kaempferol) and (D2) (drug#2, doxorubicin or cisplatin) alone that gives X% inhibition, whereas (D1) and (D2) in the numerators are the doses of (D1) and (D2) in combination that also inhibits X% (i.e. iso-effective). The (D1) and (D2) can be readily calculated from the Median effect equation of Chou et al.

\[ D_1 = D_{o1} \exp\left( \frac{f_1}{1 + f_2} \right) \]  

Where D1 is the median-effect dose obtained from the anti-log of the X-intercept of the median-effect plot, X-log (D) versus, Y = log (f1/(1-f1)) or Dm = 10^(-Y-intercept)/n, f1 is the fraction affected by dose D (e.g., 0.5 if cell growth is inhibited by 50%) and n is the slope of the median-effect plot. From (D1), (D2), and D1+D2, it becomes easy to construct an isobologram based on Eq. A: CI<1 indicates synergism; CI=1 indicates additive effect and CI>1 indicates antagonism.

For conservative mutually non-exclusive isobolograms of two agents, a third term,

\[ \frac{[D_1][D_2]}{[D_1][D_2]} \]  

Is added to Eq. A. For simplicity, the third term is usually omitted, and thus the mutually exclusive assumption or classic isobologram is indicated. In this study, the CI values obtained from the classic (mutually exclusive) calculation are given.

Fluorescence imaging
HCT-15 and MDA MB 231 cells at 60% confluency were treated with kaempferol and doxorubicin or cisplatin either alone or in combination for 24 h. At the end of treatment, cells were processed and washed with PBS, fixed in absolute alcohol for 30 min at 4 °C, rehydrated with PBS and incubated with 100 µl of propidium iodide (25 µM) at 37 °C for 5 min. Cellular destruction with the evidence of shrunken cells with condensed cytoplasm, pyknotic and fragmented nuclei was assessed under the fluorescent microscope.

Statistical analysis
The data was analysed using Microsoft Excel. Results were expressed as a means±SD-Standard deviation, where n=5. A statistically significant difference was considered to be present at p<0.05. Image J programme (NIH) was used to analyze the mean density.

RESULTS AND DISCUSSION
Effect of kaempferol on cell growth
The growth of cancer cells was inhibited in a dose-dependent manner after exposure to the phytochemical (fig. 1, 2), whereas normal human lymphocytes were not affected after exposure (fig. 3). The effect of flavonoid kaempferol on the proliferation of two cancer cell lines (HCT-15 and MDA MB 231) and normal lymphocytes were determined using MTT assay. The IC50 values for kaempferol on HCT-15 and MDA MB 231 were evaluated as 120±3.2 µg/ml and 64±1.2 µg/ml respectively (table 1). When the activity of kaempferol against cancer cells was compared with that against normal lymphocytes it was evident that kaempferol had specific anti-proliferative activity against the two cancer cell lines tested (fig. 4). Further studies were performed based on the IC50 concentrations.

Kaempferol, a flavonoid antioxidant, is an active constituent in many of the fruits and vegetables. It is one of the most commonly consumed dietary supplements [8, 10]. In recent studies, many reports demonstrated preventive and therapeutic efficacy of kaempferol in several epithelial cancer models [17, 19]. Recent epidemiological studies show that many cancer patients use alternative medicine, mostly of herbal origin. The present study shows that kaempferol is one such agent, which is nontoxic, consumed widely as a dietary supplement, and possesses strong anticancer activity against different epithelial cancers.

Fig. 1: Effect of phytochemical (kaempferol) on HCT-15, cells treated with concentrations. (1) Control; (2) 10 µg/ml; (3) 25 µg/ml; (4) 50 µg/ml; (5) 100 µg/ml and (6) 150 µg/ml. Cells were visualized under phase contrast inverted microscope (Magnification X40)
Fig. 2: Effect of phytochemical (kaempferol) on MDA MB 231, cells treated with concentrations. (1) control; (2) 5 µg/ml; (3) 10 µg/ml; (4) 25 µg/ml; (5) 50 µg/ml; (6) 100 µg/ml. Cells were visualized under phase contrast inverted microscope (Magnification X40)

Fig. 3: Effect of phytochemical (kaempferol) on lymphocytes, cells treated with concentrations. (1) control; (2) 5 µg/ml; (3) 10 µg/ml; (4) 25 µg/ml; (5) 50 µg/ml; (6) 100 µg/ml. Cells were visualized under phase contrast inverted microscope (Magnification X40)

Table 1: IC50 concentrations of kaempferol in human cancer cells HCT-15 and MDA MB 231

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Cell line</th>
<th>Concentrations (µg/ml)</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaempferol</td>
<td>HCT-15</td>
<td>5 10 25 50 100 150</td>
<td>95.04±0.70</td>
</tr>
<tr>
<td></td>
<td>MDA MB 231</td>
<td>93.50±2.21 87.68±1.1 77.93±2.85 62.66±1.54 22.07±2.19</td>
<td>37.3±1.86 64±1.2</td>
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</table>

IC50-Concentration of the compound required to inhibit cell growth by 50%, HCT-15-Human colon cancer, MDA MB 231-Breast cancer;± SD–Standard deviation, n=5

Fig. 4: Anti-proliferative activity of kaempferol on human colon cancer cells (HCT-15), breast cancer cells (MDA-MB 231) and lymphocytes

Effect of chemotherapeutic drugs on cell growth

The effects of doxorubicin and cisplatin on the proliferation of two cancer cell lines were determined using MTT assay. On both the cell lines, the chemotherapeutic drugs showed growth inhibitory effect in a dose-dependent manner. The IC50 concentrations of doxorubicin and cisplatin on HCT-15 were obtained as 49.6±0.5 µg/ml and 25.4±2.9 µg/ml respectively. When MTT assay was performed on MDA-MB 231 cells, growth inhibitory effect was observed, and IC50 concentrations were obtained at 44±1.8 µg/ml and 40.6±0.8 µg/ml for doxorubicin and cisplatin respectively (table 2).

Table 2: IC50 values of doxorubicin and cisplatin in human cancer cells HCT-15 and MDA MB 231

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Compound</th>
<th>Concentrations (µg/ml)</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-15</td>
<td>Doxo-rubicin</td>
<td>84.6±3.49 72.29±1.2</td>
<td>62.91±0.7</td>
</tr>
<tr>
<td></td>
<td>Gs platin</td>
<td>85.19±1.2 76.06±1.6</td>
<td>56.95±2.5</td>
</tr>
<tr>
<td>MDA MB 231</td>
<td>Doxo-rubicin</td>
<td>93.50±2.21 87.68±1.1</td>
<td>80.80±5.5</td>
</tr>
<tr>
<td></td>
<td>Gs Platin</td>
<td>92.94±3.26 88.26±2.19</td>
<td>77.36±2.71</td>
</tr>
</tbody>
</table>

IC50-Concentration of the compound required to inhibit cell growth by 50%, HCT-15-Human colon cancer, MDA MB 231-Breast cancer;±SD–Standard deviation, n=5

Combination effect of kaempferol and chemotherapeutic drugs on cell growth

The combination effect of kaempferol with doxorubicin or cisplatin in the HCT-15 and MDA MB 231 cell lines has been represented in CI and the results are summarised. The data was examined using median effect analysis to determine the type of interactions which occurred, i.e., antagonism (CI>1), additivity (CI=1) or synergism (CI<1) (table 3).

Table 3: CI values for HCT-15 and MDA MB 231

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell line</th>
<th>Concentrations (µg/ml)</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxo-rubicin</td>
<td>1 5</td>
<td>84.6±3.49 72.29±1.2</td>
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</tbody>
</table>

IC50-Concentration of the compound required to inhibit cell growth by 50%, HCT-15-Human colon cancer, MDA MB 231-Breast cancer;±SD–Standard deviation, n=5

The CI values for HCT-15 and MDA MB 231, when treated with kaempferol in combination with doxorubicin/cisplatin, were 0.45 and 0.65, 0.55 and 0.65 respectively, where CI<1 means synergism, CI=1 additivity, CI>1 antagonism.
means additive effects and CI>1 means antagonism. In all combination treatments, the concentrations of doxorubicin and cisplatin necessary to inhibit 50% of cancer cells (IC50) were reduced by 5 and 2.5 fold for HCT-15 and 2.4 and 2.6 fold for MDA MB 231 respectively. To assess the effect of kaempferol and doxorubicin or cisplatin on HCT-15 and MDA MB 231 cell growth, cells in the exponential growth phase were treated with different concentrations of kaempferol [5, 10, 25, 50, 100 and 150 µg/ml], doxorubicin [5, 10, 25 and 50 µg/ml] and cisplatin [5, 10, 25 and 50 µg/ml] for 24 h. At the end of the treatment, the determination of % cell death showed that these agents inhibited cell growth in a dose-dependent manner. On the basis of these results, experiments were performed on both the cell lines (for 24 h) by combining kaempferol at 30 µg/ml (1/4 IC50) with doxorubicin/cisplatin at 5, 10, 25 and 50 µg/ml for HCT-15 (fig 5). The experiment was repeated for MDA MB 231 cells by considering the concentrations of kaempferol with doxorubicin/cisplatin as 32 µg/ml (1/2 IC50) and 5, 10, 25 and 50 µg/ml respectively (fig 6). At the end of the experiments, significant synergistic growth inhibition of both HCT-15 and MDA MB 231 was observed.

The increase in systemic toxicity and drug resistance are the major drawbacks of cancer chemotherapeutic agents are led to a new challenge in the field of cancer research. To overcome this problem, extensive research has been directed towards reducing systemic toxicity and increasing drug activity in cancer therapy [2]. In this regard, combination therapy has received more attention for the purpose of finding compounds with a known mechanism of action that could increase the therapeutic index of clinical anticancer drugs [17]. The above results indicate that the \textit{in vitro} therapeutic effect of doxorubicin in terms of cell growth inhibition at the 49.6±0.5 µg/ml dose was achieved at its one-fifth concentrations (10.2±0.83 µg/ml) in combination with 30 µg/ml dose of kaempferol in HCT-15 cells whereas with cisplatin, cell growth inhibition at 25.4±2.9 µg/ml was achieved at its one-fifth concentration (10±1.34 µg/ml dose) in combination with 30 µg/ml dose of kaempferol.

However, phytochemical kaempferol and therapeutic drugs doxorubicin and cisplatin showed the cytotoxic effect on HCT-15 cells individually at 120±3.2 µg/ml, 49.6±0.5 µg/ml and 25.4±2.9 µg/ml respectively. Simultaneously, on MDA-MB 231 cell line, the \textit{in vitro} therapeutic effect of doxorubicin and cisplatin was achieved at 44±1.8 µg/ml and 40.6±0.8 µg/ml was reduced to 18±1.22 µg/ml and 15±1.87 µg/ml in combination with 32 µg/ml dose of kaempferol.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Combination</th>
<th>CI</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-15</td>
<td>1/4 IC50 kaempferol+doxorubicin</td>
<td>0.45&lt;1</td>
<td>Synergistic</td>
</tr>
<tr>
<td></td>
<td>1/4 IC50 kaempferol++cisplatin</td>
<td>0.65&lt;1</td>
<td>Synergistic</td>
</tr>
<tr>
<td></td>
<td>1/2 IC50 kaempferol+doxorubicin</td>
<td>0.55&lt;1</td>
<td>Synergistic</td>
</tr>
<tr>
<td>MDA MB 231</td>
<td>1/2 IC50 kaempferol+doxorubicin</td>
<td>0.65&lt;1</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>

Table 3: Combination index of kaempferol in combination with doxorubicin or cisplatin

CI=Combination index, HCT-15-Human colon cancer, MDA MB 231-Breast cancer

Effect of kaempferol-doxorubicin/cisplatin combination on apoptotic cell death of cancer cells

On the basis of strong growth inhibition by the synergistic effect of kaempferol-doxorubicin or cisplatin combination on HCT-15 and MDA MB 231 cells, the investigation was carried to show their effect on apoptotic morphological changes. To evaluate the effect of kaempferol and doxorubicin or cisplatin, treated cells were stained with propidium iodide. The results were compared with untreated control cells. The stained cells showed fragmentation and condensation of chromatin and other morphological features characteristic of apoptotic cells in both the cancer cells, whereas untreated control cells were not identified with nuclear alterations and showed a normal nuclear morphology characterised by diffused chromatin structure. The combinational dose concentrations of kaempferol, doxorubicin or cisplatin and treatment design were the same as that of other studies for both cell lines. As shown in (fig. 7, 8), the apoptotic cell population was increased in both HCT-15 and MDA MB 231 cell lines when treated with doxorubicin or cisplatin in combination with kaempferol.

Apoptotic death of cancer cells is considered to be a potential anti-cancer mechanism, which could control their proliferation [1, 10]. Recently, the regulation of apoptosis has been proposed as a promising target for cancer chemotherapy [19, 20]. Consistent with these reports, the data of our present study show clearly that kaempferol and doxorubicin/cisplatin combination induces strong apoptotic cell death that was ~3 fold higher than each agent alone.

Fig. 5: IC50 Concentrations of kaempferol, doxorubicin and cisplatin alone and in combination on human colon cancer cells (HCT-15). KA-Kaempferol, Dox-Doxorubicin, Cis-Cisplatin, n=5

Fig. 6: IC50 Concentrations of kaempferol, doxorubicin and cisplatin alone and in combination on human breast cancer cells (MDA-MB 231). KA-Kaempferol, Dox-Doxorubicin, Cis-Cisplatin, n=5

Fig. 7: Morphological alterations of HCT-15 cells treated with kaempferol in combination with doxorubicin/cisplatin, treated with concentrations. (1) control; (2) kaempferol (120 µg/ml); (3)-doxorubicin (50 µg/ml); (4)-cisplatin (25 µg/ml); (5)-kaempferol (120 µg/ml)+doxorubicin (50 µg/ml); (6)-kaempferol (30 µg/ml)+doxorubicin (10 µg/ml); (7)-kaempferol (120 µg/ml)+cisplatin (25 µg/ml); (8)-kaempferol (30 µg/ml)+cisplatin (10 µg/ml). Cells were visualized under fluorescence microscope (Magnification X200).
CONCLUSION
In the present investigation, we have evaluated the anti proliferative activity of kaempferol and chemotherapeutic drugs doxorubicin and cisplatin, combinational effects and nuclear alterations on the human colon (HCT-15) and breast cancer (MDA MB 231) cells. The combinational studies and fluorescent microscopic studies showed a strong synergistic therapeutic effect of kaempferol in combination with doxorubicin or cisplatin in HCT-15 and MDA MB 231 cell lines.

ACKNOWLEDGEMENT
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CONFLICT OF INTERESTS
Declared none

REFERENCES