**P53 AND CYCLIN B1 MEDIATE APOPTOTIC EFFECTS OF APIGENIN AND RUTIN IN ERα+-BREAST CANCER MCF-7 CELLS**

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**Abstract**

Tamoxifen is an effective treatment for estrogen receptor alpha positive (ERα+)-breast cancer, however patients who received the treatment for five years have greater mortality risk compared to those who did not receive tamoxifen. Furthermore, patients treated with tamoxifen developed resistance to the drug which mediated through p53 and PTEN. Therefore, the study is undertaken to determine the potential adjuvant properties of flavonoids, apigenin and rutin to promote the anticancer activity induced by tamoxifen using ERα+-breast cancer MCF-7 cell lines. MCF-7 and non-transformed breast MCF-10A cells were treated separately with apigenin, rutin, tamoxifen or the combination of each flavonoids with tamoxifen. Anti-proliferative activity and respective IC50 concentrations were determined using MTT assay. The respective IC50 concentrations obtained were used in the subsequent experiments. The anti-proliferative mechanism was determined using Annexin V-FITC morphological staining and DNA fragmentation assays. The effect on tumor suppressor (p53 and PTEN) and cell cycle related genes (p21, CDK1 and Cyclin B1) were determined by QuantiGene Plex assay. Our results showed that MCF-7 cells were more sensitive to both apigenin and rutin compared to MCF-10A cells. Both cells were sensitive to tamoxifen. Apigenin and rutin synergistically enhanced tamoxifen anti-proliferative effect in MCF-7. Meanwhile rutin protects MCF-10A against the toxicity of tamoxifen. Apigenin and rutin synergistically enhanced tamoxifen anti-proliferative effect in MCF-7. Meanwhile rutin protects MCF-10A against the toxicity of tamoxifen. Our results indicate that the anti-proliferative mechanism of apigenin and rutin is mediated by apoptosis signals. In MCF-7 cells, both tumor suppressor (p53 and PTEN) and cell cycle related genes (p21 and CDK1) were up regulated by apigenin and rutin, contrary to tamoxifen. Apigenin and rutin induced G2/M arrest and apoptosis in MCF-7 cells through p53-dependent pathway. Both flavonoids are suggested as potential adjuvant agents to enhance tamoxifen efficacy in ERα+-breast cancer treatment.

**Keywords:** Apigenin, rutin, tamoxifen, apoptosis, MCF-7
1.0 INTRODUCTION

Breast cancer is the most prevalent types of cancer diagnosed among women with 14 million new cancer cases per year as estimated in 2012 and expected to rise to 22 million annually within the next two decades [1]. Moreover, cancer deaths are predicted to rise from an estimated 8.2 million annually to 13 million per year [1]. The exact etiology of breast cancer remains unknown. However, 75% of all breast cancer patients expressed estrogen receptor alpha positive (ERα+) and therefore their development is highly dependent on estradiol. Tamoxifen competitively binds to ERα in the cancer cell, thus blocking estrogen from attaching to the receptor [2]. It has remained as the endocrine treatment for ERα+-breast cancer patients for the past 40 years [3] either in primary or metastatic stages; as well as in adjuvant therapy [4]. However, significant number of patients developed intrinsic and acquired resistances against the treatment [5]. Resistance to tamoxifen treatment in breast cancer patients is associated with suppression of tumor suppressor genes such as p53 [6] and PTEN [7].

Tumor suppressor gene p53 prevents neoplastic development by eliminating or inhibiting the proliferation of abnormal cells through cell cycle arrest or apoptosis [8]. Its mutation leading to the loss of wild-type p53 activity is frequently detected in 50% of all human cancers [9]. Evidence suggested that reactivation of p53 function would have significant therapeutic benefit [8]. p53 mutation are present in about 25% of primary breast carcinomas [10]. A correlation between the presence of p53 mutation and resistance to tamoxifen was observed in primary breast cancer patients [6]. The absence of p53 mutation seems to predict the overall patient’s survival following primary therapy [11].

Similar to p53, PTEN is also one of the most common targets of mutation in advanced stages of breast cancer progression [12]. PTEN protects p53 from survival signals by sensitizing tumor cells to p53-dependent chemotherapy [13]. Over expression of PTEN in cancer cells inhibits cell proliferation and tumorigenicity through cell cycle arrest and apoptosis [14]. In breast cancer patients, PTEN expression was lower than that of normal breast tissues adjacent to the tumors. Furthermore, its
expression correlates with the tumor size or stage. This indicates that the reduced expression of PTEN is associated with advanced stages [15].

Studies have demonstrated that the anti-proliferative effect of flavonoids is selective and more sensitive to breast cancer cells compared to normal [16]. Flavonoids such as apigenin (4',5,7-trihydroxyflavone) and rutin (3,3',4',5,7-pentahydroxyflavone-3-rutinoside) are the most abundantly occurring dietary polyphenols i.e., 60% in fruits, vegetables and seeds [17]. Apigenin inhibited the proliferation of estrogen responsive, MCF7 cells and the tamoxifen-resistant sub-lines through down regulated ERα+ and inhibited of proliferative MAPK signaling pathways. In addition, apigenin demonstrated synergistic anti-proliferative effect when combined with either tamoxifen or fulvestrant on both antiestrogen-sensitive and -resistant breast cancer cells [18]. Rutin induced G2/M phase arrest and promote apoptosis in neuroblastoma LAN-5, cells [19].

Cancer develops when the balance between cell proliferation and apoptosis is disrupted. p53 is recognized as the linkage between cyclin dependent kinases (CDKs)/Cyclins and cell cycle regulations with pro-apoptotic both Bcl-2 and Caspase families [20]. Thus, preliminary understanding on the effect of apigenin and rutin on the expression of p53, PTEN, p21 (CDK1 inhibitor), CDK1 and Cyclin B1 genes in ERα+-breast cancer cells may provide an early insight on their therapeutic potentials as anti-cancer agents; and will give a good base line to widen the possibility to extend this study into animal setting.

2.0 METHODOLOGY

Cell line

ERα+-breast cancer cells, MCF-7 were cultured in Roswell Park Memorial Institute 1640 media supplemented with 10% of fetal bovine serum and 1% antibiotics (penicillin and streptomycin) while non-transformed breast cells, MCF-10A were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 5% horse serum, antibiotics (1% penicillin and streptomycin), 0.05% hydrocortisone, 1% insulin and 0.2% recombinant human growth factor in 75 cm2 flasks. Both cells were incubated at 37°C with 5% CO2 to 80% confluent. Cells, tissue culture grade chemicals and disposable equipment were purchased from American Type Culture Collection (USA), GIBCO Invitrogen (USA) and Orange Scientific (Belgium), respectively.

Cell Treatment

Apigenin, rutin and tamoxifen were dissolved in dimethyl sulfoxide (DMSO), filtered and stored at -20°C. The final working solutions of apigenin, rutin and tamoxifen were diluted with culture media so that the final concentration of DMSO in cell culture was <0.1%. All the above chemicals were purchased from Sigma Chemical Company, USA.

Cell Proliferation

Cell proliferation was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Roche Diagnostics, Germany) according to the manufacturer’s instructions. A total of 1 x 10^4/mL cells was treated separately with apigenin and rutin at different final concentrations ranging from 10µM to 100µM for 72 hours. The combined effect between each phytochemicals with tamoxifen at respective IC50 values was determined. Tamoxifen was used as a positive control. Cells cultured in complete media with 0.1% of DMSO were used as negative controls.

Cell Apoptosis

Anti-proliferative mechanism was determined using Annexin V-FITC apoptosis detection assay (Sigma-Aldrich, USA) according to the manufacturer’s instructions. A total of 1 x 10^4/mL cells were treated separately with apigenin and rutin at respective IC50 concentration obtained from proliferation assay for 72 hours. Again, tamoxifen and cells cultured in complete media with 0.1% of DMSO were used as positive and negative controls, respectively. Cells were washed twice with phosphate buffer saline (PBS), detached by accutase, collected by centrifugation and re-suspended in ice-cold binding buffer. After staining with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V (Annexin V-FITC), cells were analyzed using flow cytometry (BD Bioscience Pharmingen, USA) to distinguish between viable (Annexin V-FITC−/PI−), early apoptotic (Annexin V-FITC+/PI−), late apoptotic (Annexin V-FITC+/PI+) and necrotic cells (Annexin V-FITC−/PI+). PBS and accutase were purchased from Sigma Chemical Company (USA).

DNA fragmentation analysis (Cellular DNA fragmentation ELISA: Roche Diagnostics, Germany) was conducted to discriminate between late apoptotic and primary necrotic cells. Cells were treated with the same dose and duration as the morphological staining assay above.

Gene Expression Analysis

The expression of target genes in both flavonoids (apigenin or rutin) and tamoxifen-treated cells against untreated after normalization with ribosomal protein L13A (RPL13A) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) housekeeping genes were determined using QuantiGene 2.0 Plex assay (Affymetrix, USA). A total of 1 x 10^4/mL cells were treated separately with apigenin and rutin at respective IC50 concentration for 72 hours. Similar with the previous assays, tamoxifen and cells cultured in complete media with 0.1% of DMSO were used as
positive and negative controls, respectively. The assay was conducted following the protocol described in QuantiGene 2.0 Plex User Manual.

**Statistical Analysis**

Data were expressed as mean ± standard deviation. Each experiment was repeated three times, independently and in triplicates. One-way ANOVA test was used to compare the mean of each group. Statistical significant was set up at p<0.01.

### 3.0 RESULTS AND DISCUSSION

**MCF-7 Cells were More Sensitive to Apigenin and Rutin Compared to MCF-10A**

The effect of apigenin and rutin on cells proliferation of ERα⁺-breast cancer and non-transformed breast cells (MCF-7 and MCF-10A, respectively) at different concentrations (10µM to 100µM) for 72 hours were investigated (Figure 1a) and respective IC₅₀ concentrations were summarized in Table 1.

![Figure 1a](image)

**Figure 1a** The effect of apigenin, rutin and tamoxifen on ERα⁺-breast cancer, MCF-7 (A) and non-transformed breast, MCF-10A (B) cell proliferation, respectively. Data were mean ± SD of three independent experiments and in triplicates. *p<0.01 compared with respective untreated control group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC₅₀ concentrations (µM)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Apigenin</td>
<td>34.34±0.01**</td>
</tr>
<tr>
<td>Rutin</td>
<td>46.09±0.01**</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td><em>30.66±0.01</em></td>
</tr>
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</table>

IC₅₀ is the concentration that inhibits cellular proliferation by 50%. Data were mean ± SD of three independent experiments and in triplicates. *p<0.01 compared with respective untreated control group; p<0.01 compared with rutin in MCF-7 cells; p<0.01; p<0.01 compared to tamoxifen in MCF-7 cells; ++p<0.01 compared with tamoxifen in MCF-10A cells; *p<0.01 compared between rutin treatment group in MCF-7 and MCF-10A cells.

Apigenin and rutin selectively inhibited the proliferation of MCF-7 cells with IC₅₀ of 34.34±0.01µM and 46.09±0.01µM, respectively at 72 hours. No IC₅₀ was obtained for MCF-10A cells even after treated with apigenin up to 100µM. Rutin IC₅₀ for MCF-7 cells (46.09±0.01) is much lower compared to MCF-10A (94.14±0.01µM). It seems that MCF-7 cells are more sensitive to apigenin and rutin compared to MCF-10A cells. In contrast, tamoxifen was non-selective to both cells with IC₅₀ of 30.66±0.01µM and 38.17µM, respectively.

**Tamoxifen Anti-proliferative Effect on MCF-7 Cells were Enhanced by Apigenin and Rutin**

The combined effects of tamoxifen with apigenin and rutin at respective IC₅₀ concentrations after 72 hours of treatments as summarized in Figure 1b.

![Figure 1b](image)

**Figure 1b** Effects of tamoxifen combination with apigenin and rutin (IC₅₀ concentrations) on ERα⁺-breast cancer, MCF-7 and non-transformed breast, MCF-10A cells proliferation. Data were mean ± SD of three independent experiments and in triplicates. *p<0.01 compared with respective untreated control group; p<0.01 compared between the combined treatment of apigenin and tamoxifen group with tamoxifen alone in MCF-7 cells; **p<0.01 compared between the combined treatment of rutin and tamoxifen group with tamoxifen alone in MCF-10A cells.
Tamoxifen anti-proliferative effect against MCF-7 cells was more pronounced in the respective combined treatment with apigenin (67.66±2.22% inhibition at p<0.01, n=3) and rutin (70.21±2.95% inhibition at p<0.01, n=3) compared to tamoxifen alone (51.55±2.31% inhibition at p<0.01, n=3). Interestingly, tamoxifen anti-proliferative effect on MCF-10A cells was reduced in the combined treatment with rutin (21.81±1.70 at p<0.01, n=3) compared to tamoxifen alone (54.35±4.87 at p<0.01, n=3) (Figure 1b). The combined effect of tamoxifen with apigenin on MCF-10A cells was not determined as no IC50 concentration for apigenin was obtained.

Apigenin and rutin are naturally occurring polyphenolic compounds of flavonoids. Polyphenols interfere with breast and ovarian carcinogenicity through modulation of estrogen receptor activities [21] due to their structural similarity with 17 β-oestradiol estrogen receptor subtypes α and β [22]. Furthermore, polyphenols reduced the progression of these cancers through enhancement of p53-mutant [19], cell cycle arrest [23] and activation of apoptosis [23].

Using respective IC50 concentrations, ERα+-breast cancer, MCF-7 cells were more sensitive to both apigenin and rutin compared to non-transformed breast, MCF-10A cells. On the contrary, both cells were sensitive to tamoxifen (Table 1). These flavonoids appeared to have the ability to enhance tamoxifen anti-proliferative effect towards MCF-7 while protecting MCF-10A against tamoxifen (Figure 1b). Flavonoids such as apigenin, rutin and genistein (4',5,7-trihydroxyisoflavone) were cytotoxic to neuroblastoma LAN-5 and ovarian SiHa cancer cells [19][24]. However, normal cells were less sensitive to flavonoids [25]. Furthermore, apigenin and genistein were able to sensitize estrogen receptor negative (ER-) breast MDA-MB-453; and ovarian A2780 and C200 cancer cells compared to 5-fluorouracil and cisplatin, respectively [26][27][28]. Contrary to both flavonoids, 4-hydroxytamoxifen was cytotoxic to both MCF-7 and MCF-10A cells [29].

The results of cell apoptosis induced by apigenin and rutin compared to tamoxifen at 72 hours are represented in Figure 2a and summarized in Figure 2b. Apigenin, rutin and tamoxifen treatments in MCF-7 cells resulted in total apoptotic population of 48.40±2.41% (p<0.01, n=3), 47.60±1.67% (p<0.01, n=3) and 51.05±0.62% (p<0.01, n=3) respectively, compared to untreated control cells (1.45±0.03 at p<0.01, n=3) as shown in Figures 2a and 2b. Rutin treatment only resulted in 13.07±0.76% (p<0.01, n=3) of total apoptotic population in MCF-10A cells compared to tamoxifen, 45.73±2.94% (p<0.01, n=3) even though rutin IC50 concentration was thrice higher than tamoxifen. Apoptosis was further confirmed by using DNA fragmentation assay (Figure 3). The effect of apigenin on MCF-10A apoptosis could not be determined as no IC50 concentration was obtained.

Figure 3 shows the results of apoptotic DNA fragmentation in ERα+-breast cancer, MCF-7 cells (A) and non-transformed breast, MCF-10A cells (B) induced by flavonoids and tamoxifen at respective IC50 concentrations. DNA fragmentation was detected in MCF-7 cells after treated with apigenin, rutin and tamoxifen for 72 hours at respective IC50 concentrations. In MCF-10A, DNA fragmentation was not detected at 72 hours in rutin and tamoxifen treatments. It was detected later, at 84 hours and 96 hours, respectively. Consistent with the anti-proliferative effect, higher apoptosis was observed in rutin-treated MCF-7 cells compared to MCF-10A. Apoptosis was equally high in both cells after tamoxifen treatment (Figure 3). Apigenin reduced the proliferation of prostate cancer PC-3, and DU145 cells through apoptosis, but was less sensitive to normal prostate PWR-1E cells [30]. It caused G1 phase arrest and apoptosis to cervical cancer HeLa cells [31]. Anti-neuroblastoma effect of rutin on LAN-5 was induced via G2/M phase arrest and apoptosis [20]. Consistent with its cytotoxicity effects, 4-hydroxytamoxifen enhanced DNA damage in both MCF-7 and MCF-10A cells [32].

**Apigenin and Rutin Treatments Lead to Apoptosis**

To examine whether cells undergo apoptosis; untreated, flavonoids-treated (apigenin or rutin) and tamoxifen-treated breast cancer MCF-7 and non-transformed breast MCF-10A cells were stained with Annexin V-FITC and PI at respected IC50 concentrations.

Flow cytometry analysis of PI and Annexin V-FITC stained cells are a standard procedure to distinguish early apoptotic cells (Annexin V-FITC+/PI−) and late apoptotic cells (Annexin V-FITC+/PI+). However, this method could not discriminate between the late apoptotic cells with primary necrotic cells, since both of these groups of cells are Annexin V-FITC+/PI+. These two groups are able to be distinguished by DNA fragmentation method [29].
Figure 2a A representative flow cytometry analysis of flavonoids (apigenin and rutin) and tamoxifen treatments on ERα+ breast cancer, MCF-7 cells (A) and non-transformed breast, MCF-10A cells (B) at respective IC50 concentrations. The percentage of total apoptotic population from the above scatter plot graphs obtained from 3 set of experiments was presented in bar graph (Figure 2b).

Figure 2b Bar chart analysis representing total apoptotic population in MCF-7 and MCF-10A cells obtained from flow cytometry analysis. All treatment increased the proportion of total apoptotic population in MCF-7 cells. Rutin induced 3.5 times less apoptosis than tamoxifen in MCF-10A cells. Data were presented as mean ± SD of three independent experiments and in triplicates. *p<0.01 compared with respective untreated control group; †p<0.01 compared between rutin treatment group in MCF-7 with MCF-10A cells.

Figure 3 DNA fragmentation analysis of flavonoids and tamoxifen treatments on ERα+ breast cancer, MCF-7 (A) and non-transformed breast, MCF-10A cells (B). Data were presented as mean ± SD of three independent experiments and in triplicates. *p<0.01 compared with respective untreated control group; †p<0.01 compared between apigenin treated group with rutin in MCF-7 cells; ‡p<0.01 compared between apigenin treated group with tamoxifen in MCF-7 cells; *p<0.01 compared between rutin treated group with tamoxifen in MCF-10A cells.
Apigenin and Rutin Up Regulate p53 and Down Regulates Cyclin B1 in MCF-7 Cells

In order to investigate the pathway by which apigenin and rutin induced apoptosis, gene expression analysis for tumor suppressors and cell cycle related genes was performed. Target genes were normalized with RPL13A housekeeping gene and summarized in Table 2.

Results show increased expression of p53, PTEN, and p21 in response to apigenin and rutin treatments compared with control group (p<0.01, n=3). No changes in CDK1, but Cyclin B1 was evidently decreased (p<0.01, n=3). Contrary to tamoxifen, PTEN, p21 and Cyclin B1 were decreased (p<0.01, n=3), but no changes to p53 and CDK1 (Table 2). Similar pattern for all target genes were obtained after normalization with HPRT housekeeping gene.

Table 2: Effect of apigenin and rutin on the expression of tumor suppressors and cell cycle related genes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor suppressor gene</th>
<th>Cell cycle related gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p53</td>
<td>PTEN</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Apigenin</td>
<td>1.75</td>
<td>10.29</td>
</tr>
<tr>
<td>±0.94* ±1.29*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rutin</td>
<td>1.52</td>
<td>13.47</td>
</tr>
<tr>
<td>±0.13* ±0.59*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>0.99</td>
<td>0.63</td>
</tr>
<tr>
<td>±0.06 ±0.09*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The mRNA expression of p53, PTEN, P21, CDK1, Cyclin B1 in MCF-7 treated with apigenin, rutin and tamoxifen at respective IC50 concentrations for 72 hours. The levels of each gene mRNA was normalized to the value of the RPL13A housekeeping gene. Results obtained from three independent experiments are expressed as mean ± SD. Value of 1.00 is indicated as no changes, above 1.00 as up regulated and below 1.00 as down regulated. *p<0.01 compared with untreated control group.

Apigenin and rutin are suggested to affect multiple cell signaling processes in cancers cells which arrest their cell cycle progression and induction of apoptosis [32][33][34]. Apigenin and rutin induction of apoptosis in MCF-7 cells may be associated with increased expression of p53, PTEN and p21; and decreased cyclin B1 genes. Tamoxifen decreased the expression of PTEN, p21 and Cyclin B1 genes, while no changes in p53 was observed (Table 2). These results suggest that both flavonoids arrested MCF-7 cells at G2/M and both the arrest and apoptosis actions on MCF-7 cells are p53-dependent, contrary to tamoxifen. Accordingly, apigenin G1 phase arrest and induction of apoptosis in HeLa cells was p53 dependent and associated with increased expression of a CDK inhibitor, p21; and apoptotic mediated Fas/APO-1 and caspase-3 proteins. Apigenin also decreased the expression of Bcl-2 protein, an anti-apoptotic factor [33]. Apigenin arrested the growth of HT-29 and SW620 colon cancer cells at G2/M phase which were suggested mediate by inhibition of CDK1 protein [34]. Interestingly, apigenin is able to increase the stability of p53 gene in normal cells [36]. In addition, rutin G2/M arrest and increased in apoptotic rate in neuroblastoma LAN-5 cells was associated with reduced Bcl-2/Bax ratio [20]. Decreased of p53 expression in rat CCI4-induced hepatotoxicity was restored by rutin [35].

4.0 CONCLUSION

In conclusion, apigenin and rutin anti-proliferative effect on ERα+ breast cancer MCF-7 cells could be mediated through p53-dependent-G2/M phase arrest and apoptosis. It appears that apigenin and rutin are able to synergistically enhanced tamoxifen efficacy on MCF-7 cells and are potential candidates as adjuvant agents in reducing tamoxifen adverse effects.

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References


