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Original Research Article

Apigenin exerts anticancer effects on human cervical cancer cells via induction of apoptosis and regulation of Raf/MEK/ERK signalling pathway

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Abstract

Purpose: *To investigate the anticancer activity of apigenin on human cervical cancer cells.*
Methods: *The anti-proliferative effects of apigenin on HeLa cervical cancer cells were determined by 3-(4,5-dimethylthiazol-2-yl)-)-2,5-diphenyltetrazolium bromide (MTT) and clonogenic assays, while its effect on apoptosis was assayed by DAPI and annexin V/PI double staining. Expression of proteins was assessed by immunoblotting.*
Results: *Apigenin exerted anticancer effects on HeLa cervical cancer cells with an IC50 of 15 µM, and also reduced the colony formation of HeLa cells. These antiproliferative effects were due to induction of apoptosis as indicated by DAPI and annexin V/PI staining. Apigenin altered Bax/Bcl-2 ratio, thereby triggering apoptosis, and also inhibited the Raf/MEK/ERK signalling pathway.*
Conclusion: *These results indicate that apigenin suppresses the growth of cervical cancer cells and may prove to be an important molecule for the treatment of cervical cancer.*

Keywords: *Cervical cancer, Apigenin, Apoptosis, Bax*

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INTRODUCTION

Cervical cancer is one of the most reported cancers among women around the globe [1]. Annually, approximately 500,000 women are diagnosed with cervical cancer which constitutes about 9% of all newly-diagnosed cancers [1,2]. The conventional treatments for cervical cancer, such as radical hysterectomy and radiotherapy have a lot of adverse effects on the overall health of the patients [3,4].

Naturally-occurring compounds have gained considerable attention in the treatment and prevention of various types of cancers [5-7]. Flavonoids constitute a diverse group of polyphenolics with a benzo-γ-pyrone skeleton and are widely distributed in the plant kingdom [8]. These are also frequently found in fruits, grains, green tea and other dietary supplements [9,10]. Numerous biological activities have been reported for flavonoids. These include antioxidant, antitumor, anti-inflammatory, anti-allergenic and hepatoprotective effects [11,12].

Studies carried out previously suggest that diets rich in flavonoids are associated with decreased risk of cancers such as colon and prostate cancers [13,14]. Flavonoids such as flavopiridol, epigallocatechin gallate and quercetin have emerged as potent anticancer drug candidates, and some of them have already entered the stage of clinical trials [15]. Apigenin is a naturally-occurring flavonoid known to possess many pharmacological properties such as anti-inflammatory, antioxidant, and anticancer effects [15]. The present study was designed to investigate the anticancer activity of apigenin against HeLa cervical cancer cells.

EXPERIMENTAL

Antiproliferative assay

The viability of the cells was investigated by MTT assay. Cultured HeLa cervical cancer cells were grown at a density of 1.5×10^4 in 96-well microtiter plates, and subjected to treatment with apigenin (0-100 µM). This was followed by the addition of MTT solution to all the wells. The absorbance of the well plates was read at 570 nm in an ELISA plate reader.

Clonogenic assay

To assess the impact of Mukonal on the colony-forming potential of apigenin, the HeLa cells were harvested at exponential phase and counted using a hemocytometer. The platting of the cells was carried out at 200 cells /well. The plates were then kept at 37 °C for 48 h to permit cell adherence. This was followed by the addition of different concentrations of apigenin (0, 7.5, 15 and 300 µM). Following treatment with Mukonal, the cell plates were again incubated for 6 days, and thereafter washed with PBS and fixed with methanol. The HeLa cells were then stained with crystal violet and examined by microscopy.

Apoptosis assay

The HeLa cervical cancer cells were grown in 6-well plates (2×10^5 cells per well) for 24 h, and treated with apigenin at 0, 7.5 and 30 µM doses for 24 h. Thereafter, the cells were DAPI-stained to detect apoptosis by fluorescence microscopy as previously reported [9]. The percentage of apoptotic cells was determined using an FITC-Annexin V/PI Apoptosis Detection Kit as per the instructions of the manufacturer.

Western blotting analysis

The HeLa cervical cells were lysed with ice-cold hypotonic buffer. After estimating the protein contents in each of the cell extracts, the samples containing the proteins were loaded and separated on SDS–PAGE. Subsequently, transference of the gels to a nitrocellulose membrane and incubation with the primary antibody (1:1000) for 24 h at 4 °C were done. Thereafter the membrane was incubated with HRP-conjugated secondary antibody (1:1000) at 24 °C for about 1 h. and the protein bands were visualised by enhanced chemi-luminescence reagent.

Statistical analysis

The assays were carried out in triplicate and the results presented as mean ± SD. Data were analysed for significant differences using one way ANOVA and Tukey's test with the aid of GraphPad Prism 7 software. Values of $p<0.01$ were taken as indicating statistical significance.

RESULTS

Anticancer effects of apigenin on HeLa cervical cancer

The results of the MTT assay revealed that apigenin (Figure 1) displayed potent anti-proliferative effect against HeLa cells in a dose-dependent manner, with an IC_{50}15µM (Figure 2). Furthermore, it was revealed that apigenin treatment reduced the number colonies formed by viable HeLa cells in a dose-dependent fashion (Figure 3), indicating its anti-proliferative effects.

Apigenin caused apoptosis in cervical cancer HeLa cells

After treatment with the different concentrations of apigenin, apoptosis was determined by DAPI staining. The results showed that apigenin caused apoptosis in a concentration-dependent pattern, as evident from the greater density of white color nuclei (Figure 4). The apoptotic ell populations were further estimated by annexin V/PI double staining. The results showed that the apoptotic cell populations increased from 2.35 % in the control group, to 38.54% at 30µM apigenin (Figure 5). In addition, apigenin increased the expression of Bax, and decreased the expression of Bcl-2 (Figure 6).

Apigenin inhibits the Raf/MEK/ERK signalling pathway

The effect of apigenin on the Raf/MEK/ERK signal transduction pathway was also investigated and the results revealed that apigenin inhibited the (phosphorylated) p-MEK and p-ERK dose dependently (Figure 7).

However, no effects were observed on MEK and ERK.

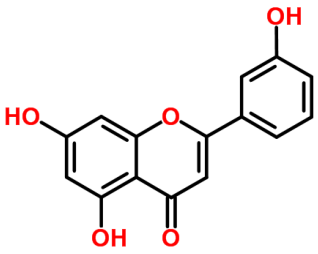

Figure 1: Chemical structure of apigenin

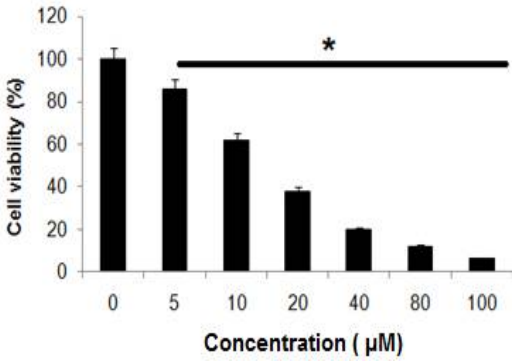

Figure 2: Effect of apigenin on the viability of HeLa cells. The experiment was performed in three replicates. Values are mean ± SD (*p < 0.01)

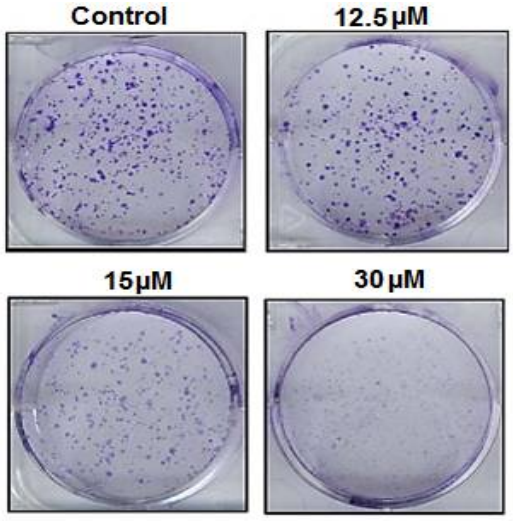

Figure 3: Dose-dependent effect of apigenin on the colony formation potential of HeLa cells. The experiment was performed in three replicates

DISCUSSION

Cervical cancer is one among the most commonly diagnosed cancers in women the world over. Indeed, it is estimated that about 500,000 women are diagnosed with cervical cancer annually [1,2]. The treatment options for cervical cancer are limited and inefficient, and they are associated with a lot of adverse effects. Hence, there is need for the exploration of new molecules for the treatment of cervical cancer [2]. Apigenin is a naturally-occurring flavonoid reported to possess many biological activities such as antioxidant and anti-tumor activities [15].

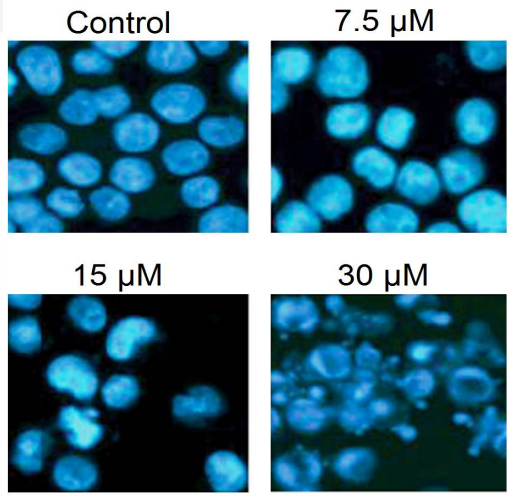

Figure 4: Effect of apigeninon apoptosis in HeLa cancer cells as evident from the DAPI staining. The experiment was performed in three replicates

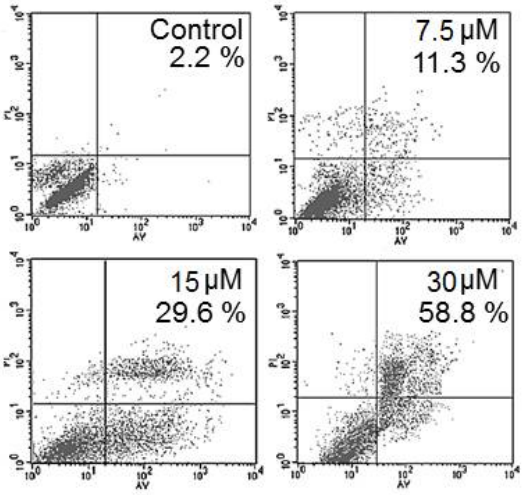

Figure 5: Effect of different doses of apigenin on percentage of apoptotic HeLa cancer cells, as determined using annexin V/PI staining. The experiment was performed in three replicates

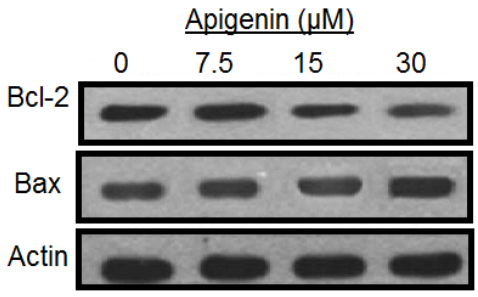

Figure 6: Effect of indicated doses of apigenin on Bax and Bcl-2 proteins as revealed through western blots. The experiment was performed in three replicates

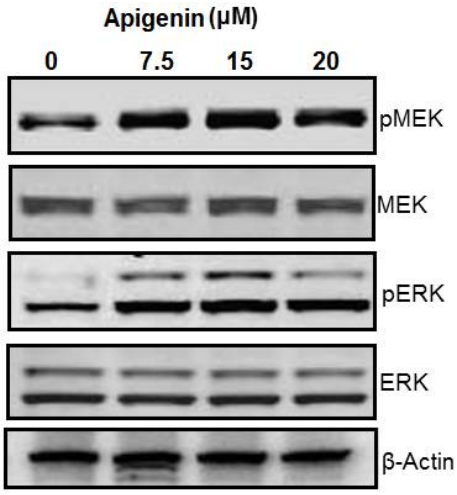

Figure 7: Effect of indicated apigeninon Raf/MEK/ERK signalling pathway

In this context, the anticancer activity of apigenin against Hela cervical cancer cell line was evaluated. It was observed that apigenin exhibited potential growth-inhibitory effects on HeLa cells, as evident from proliferation assay. The results of MTT assay were further complimented and validated by the colony formation assay. As it has been shown previously, several anticancer drugs trigger antiproliferative effects through induction of apoptosis. For example, the anticancer drugs, such as taxol have been shown to activate apoptotic cell death and cause DNA damage [16-18]. To assess if apigenin triggers apoptosis in HeLa cells, the apigenin-treated cells were DAPI-stained. It was revealed that apigenin induced apoptotic cell death concentration-dependently.

Furthermore the results of annexin V/PI staining revealed that the percentage of the apoptotic cell populations increased with increase in the concentration of apigenin which was concomitant with the enhancement of Bax expression and decline in Bcl-2 expression. The expressions of Bax and Bcl-2 shift the cells towards apoptosis. Previously it has been reported that the Bax/Bcl-2 ratio is a vital factor for the induction of apoptosis [19]. The Raf/MEK/ERK signalling pathway is involved in the proliferation of cancer cells, and drugs that target this pathway are considered important [20]. In the present study it was observed that apigenin downregulated the expression of p-MEK and p-ERK in a concentration-dependent manner. These inhibitory effects of apigenin on cervical cancer cell proliferation may prove beneficial in the treatment and management of cervical cancers.

CONCLUSION

The results obtained in this study indicate that apigenin exerts anticancer effects on cervical cancer cells via induction of apoptosis and inhibition of Raf/MEK/ER signalling pathway. This confirms the potential of apigenin as anticancer agent. Thus, this flavonoid may be utilised as a lead molecule in the development of anticancer chemotherapy.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Jie Yang and Jing Fa performed all the experiments under the supervision of Bingxing Li. Bingxing Li designed the study.

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