Original article

Daidzin inhibits growth and induces apoptosis through the JAK2/STAT3 in human cervical cancer HeLa cells

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ABSTRACT

Daidzin, 4′, 7-dihydroxyisoflavone is an isoflavonic phytoestrogen present in leguminous plants. Traditional Chinese medicine utilizes daidzin to treat various diseases such diarrhea, fever, hepatitis, cardiac problems etc. In current study we examined the anticancer activity of daidzin against human cervical cancer in vitro. HeLa, human cervical cancer cell line was purchased from ATCC and the cells were cultured with DMEM medium. The cytotoxic effect of daidzin against HeLa cell line was analyzed with MTT assay. The IC-50 value was obtained at 20 μM hence the cells were treated with 20 μM of daidzin for further analysis. ROS generation was assessed with DCFH-DA staining and the induction of apoptosis was examined with Rhoadmine-123 staining. Acridine orange and ethidium bromide staining was done to examine the apoptotic and viable cells. Further the matrigel cell adhesion assay was done to analyze the inhibitory property of daidzin against cancer cell adhesion. Apoptotic induction of daidzin was examined by estimating the levels of Caspase 8 & 9 using ELISA technique. Inflammatory and cell proliferation signaling proteins were analyzed with qPCR analysis to confirm the anticancer activity of daidzin against human cervical cancer HeLa cell line. Daidzin significantly generated ROS and altered the mitochondrial membrane permeability in HeLa cell line. The results of AO/EtBr staining prove daidzin induced apoptosis in HeLa cell line and it also inhibited the cell adhesion property of HeLa which is reported in our matrigel cell adhesion assay. It also increased the caspases 8 & 9 which are key regulators of apoptosis. Daidzin significantly decreased the expression of inflammatory gene and cell proliferating signaling molecule. To, conclude our results confirm daidzin effectively decreased inflammation and induced apoptosis in human cervical cancer HeLa cell line.

1. Introduction

One of most prevalent type of cancer which affects women globally is cervical cancer. Compared to developed countries women in underdeveloped countries are major victims of cervical cancer (Schiffman, 2017). Every year about 3.5 lakh new cases of cervical cancer were reported ant it accounts 10% of all cancers in women (Di Felice et al., 2015). Even though it is preventable and can be treated if diagnosed at early stage, it accounts to be the major cause for disability adjusted life years in most of the patients (Ferlay et al., 2015). Currently cervical cancer is treated with hysterectomy, chemotherapy and radiotherapy which render numerous side effects. Hence, there is need to discover a novel anticancer drug which effectively treats cervical cancer without causing any side effects.

Polyphenols are water soluble organic compound present abundantly in plants. Various studies have proved polyphenols has protective effect against chronic diseases (Lecour & Lamont, 2011). Epidemiological studies also reported polyphenols prevents cancer. Natural polphenols such as genistein, luteolin, resvertrol, apigenin, quercetin have reported to have anticancer effect against various malignant cells (Singh et al., 2017; Sharma et al., 2018).

Daidzin, 4′, 7-dihydroxyisoflavone is highly consumed polyphenol present in leguminous plants, fruits and nuts (Liggins et al., 2002). Daidzin is reported to have anticancer activity against prostate cancer cells (Hsu et al., 2010), breast cancer (Choi & Kim, 2008), colon and hepatic cancer (Park et al., 2013a,b). Daidzin also demonstrated the antiepileptic activity (Kazmi et al., 2020). Daidzin resembles the structure of mammalian estrogen and it is

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reported to be regulate estrogenic activity in diseases such breast cancer, osteoporosis, cardiovascular diseases (Vitale et al., 2013). Hence in the current study we analyzed the protective effect of daidzin against cervical cancer in vitro. HeLa cell line were treated with daidzin and examined for the cytotoxic, apoptotic, anticancer activity of daidzin against human cervical cancer HeLa cell line.

2. Materials & methods

2.1. Chemicals

Daidzin, doxorubicin, dichlorofluorescin diacetate, 4,6-diamidino-2-phenylindole dihydrochloride, Rhodamine 123 stain, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and tryphan blue were procured from Sigma Aldrich, USA. Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Antibiotic solution were purchased from Hi-Media Laboratories.

2.2. Culturing of HeLa cells

HeLa, human cervical cancer cell line procured from ATCC, USA and utilized for the current study. The cells were cultured using Eagle's Minimum Essential medium along with 10% fetal bovine serum and 1% penicillin–streptomycin solution. The cells were incubated at 37 °C, 5% CO_2 and the culture medium were replaced for every 48 h. Upon attaining 80% of confluence the cells were subcultrured using 0.25% trypsin-EDTA solution and the subcultured cells were used for further experiments.

2.3. MTT assay

HeLa cells were seeded on to a 96 well culture plate and incubated for 24 h at 37 °C, 5% CO_2. After the incubation period the cells were treated with different concentrations of daidzin ranging from 0 to 45 μM/ml (0, 5, 10, 15, 20, 25, 30, 35, 40, 45 μM/ml) for 24 h. The cells were then subjected to MTT assay, the medium was carefully discarded. To each well 50 μl of serum free culture medium and 50 μl of MTT solution were added, the cells were incubated at 37 °C in dark for 3 h. After incubation, 150 μl of MTT solvent was added to each well. The plates were wrapped with foil and placed on orbital shaker for 15 min. The optical density of the solution was quantified at 590 nm using Shimadzu UV spectrophotometer (Tominaga et al., 1999). Percentage of viable cells was calculated using the below mentioned formula. The experiments were carried into triplicates.

$$Percentage of viable cells = \frac{Optical Density of the test sample}{Optical Density of the controls sample} \times 100\%$$

2.4. DCFH-DA staining

HeLa cells were cultured in 24 well plates and utilized for DCFH-DA staining. The cells were then treated with 20 μM daidzin and doxorubicin, incubated in a 5% CO_2 incubator for 24 h at 37 °C. After incubation period the cells were stained with 10 μM DCFH-DA stain and incubated in dark for 30 min. Using PBS the cells were rinsed twice and examined for fluorescent emitting cells under microscope (Olympus).

2.5. Rhodamine-123 staining

The mitochondrial membrane permeability of daidzin treated and untreated cells were assessed using Rhodamine 123 staining technique. HeLa cell were cultured in 24 well plates and treated with 20 μM daidzin. As positive control the cells were treated anti-cancer drug doxorubicin and incubated for 24 h. After incubation period the cells were rinsed with PBS twice and treated with 1mMol Rhodamine 123 stain. The cells were incubated for 15 min in dark. The stained cells were then viewed under fluorescent microscope and images were captured to assess fluorescent cells.

2.6. Acridine Orang/Ethidium bromide (AO/EtBr) staining

Apoptosis induction by daidzin on human cervical cancer HeLa cell line was examined with dual staining technique using AO/EtBr stains. HeLa cells were treated with 20 μM daidzin and doxorubicin, incubated for 24 h in 5% CO_2 incubator at 37 °C. The treated cells were then rinsed with PBS and stained with acridine orange and ethidium bromide mixture (1:1). The cells were incubated in dark for 30 min, after incubation the stained cells were viewed and photographed under fluorescent microscope.

2.7. Cell adhesion assay

HeLa cell line treated with daidzin and doxorubicin was subjected to matrigel gel cell adhesion assay to assess the cell adhesion property of HeLa cells after daidzin treatment. The cells were suspended in DMEM medium (serum free) and seeded on matrigel coated plates incubated for 2 h. After 2 h, using PBS the cells were rinsed thrice to remove unattached cells. The remaining adherent cells were subjected to methanol fixing for 10 min and then fixed cells were stained with methylene blue stain for 15 min. The cells were then observed and assessed under microscope (Olympus).

2.8. Estimation of caspases activity

Caspase 8 and Caspase 9 activity in daidzin treated HeLa cell line were assessed using ELISA kits (Biocompare, USA). The ELISA kits were purchased from ThermoFischer Scientific, USA and the test was performed according to the manufacturer's protocol. The optical density of final product was quantified at 450 nm with ELISA microplate reader. The concentration of each sample was interpolated from the standard curve plotted with subjective OD to the standard concentrations.

2.9. qPCR analysis

HeLa cell lines were treated with daidzin and doxorubicin, incubated for 24 h at 37 °C with 5% CO_2. After incubation period the cells were subjected to total RNA isolation using TriZol reagent. The cells were sonicated with TriZol reagent and the cell lysate was collected. 1 ml of cell lysate was treated with 500 μl and subjected to centrifugation at 10000 rpm for 15 min at 4 °C. The supernatant was collected and equal volume of ice cold isopropanol was added to each sample. The sample was incubated for 2 h to precipitate RNA and was subjected to centrifugation for 10 min at 10000 rpm at 4 °C. The pellet was collected and rinsed with 70% ethanol twice. The pellet was collected air dried and dissolved with sterilized milliQ water. The sample was further subjected to RNA quantification with NanoDrop spectrophotometer. Further RNA was converted to cDNA using the HiScript II Q RT SuperMix kit (Vazyme, China) for real time qPCR analysis.

The gene expression inflammatory cytokines TNF-α, NFκB, IL-6, COX-2 and cell proliferating gene JAK-2, ERK and STAT were estimated by amplifying the above genes using SYBR green master mix. The results were analyzed using comparative CT method $(2 - \Delta\Delta Ct)$ and the fold change of samples were calculated using

2.10. Statistical analysis

All the experiments were done thrice and the data were statistically analyzed with GraphPad Prism version 6.02 software. The data were analyzed with One Way ANOVA followed by post hoc Duncan's test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Daidzin induces cytotoxicity in HeLa cell line

Fig. 1 depicts the cytotoxicity assay results of HeLa cells treated with different concentrations of daidzin ranging from 0 to 100 μM/ml (0, 2.5, 5, 10, 15,25, 50,75 and 100 μM/ml) for 24 h. Daidzin significantly decreases the cell viability of HeLa cells in a dose dependent manner. The IC50 value was obtained at the dose of 20 μM treated cells and 50 μM treated cells shown<20% of viable cells.

3.2. Daidzin induces ROS generation in HeLa cell line

Reactive oxygen species generated by daidzin in HeLa cells were assessed with DCFH-DA staining and the results were illustrated in the Fig. 2. Fig. 2A control cells shown decreased number of fluorescent cells whereas daidzin (B) and doxorubicin (C) treated cells shown increased number of fluorescent cells

4. Daidzin impairs mitochondrial membrane permeability in HeLa cell line

Increased mitochondrial membrane permeability was considered to be a key event in induction of apoptosis. Hence the mitochondrial membrane permeability of daidzin treated and untreated cells were examined using Rhodamine 123 staining technique and the results were depicted in Fig. 3. There is a decrease number of fluorescent observed in daidzin treated cells (B) which comparatively equal to the number of cells observed in doxorubicin treated cells (C). Control cells shown increased fluorescence (A).

4.1. Daidzin induces apoptosis in HeLa cell line

Fig. 4 illustrates the results of AO/EtBr stained HeLa cell line treated with daidzin and doxorubicin. Control cells shown increased green fluorescence with intact nucleus (A), whereas
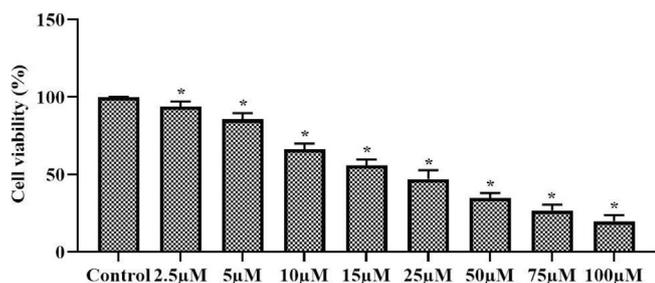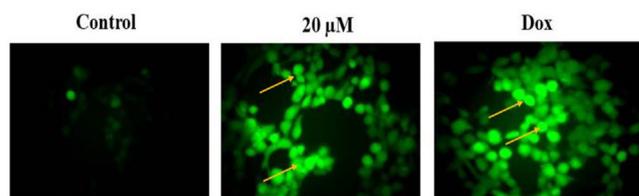

Fig 2. Daidzin induces oxidative stress in HeLa cell line. Human cervical cancer HeLa cell lines were treated with daidzin and doxorubicin for 24 h. The cells subjected to DCFH-DA staining and the representative stained cells of each group were depicted. Control (A), daidzin treated cells (B) and doxorubicin treated cells (C). The increased green fluorescence (yellow arrows) in the daidzin and Dox treated cells demonstrated the elevated ROS accumulation in the HeLa cells.
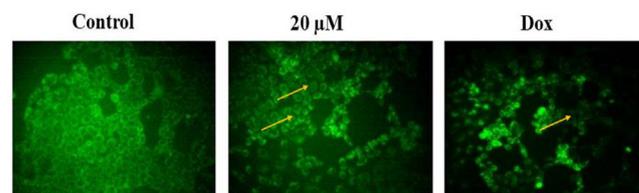

Fig 3. Daidzin impairs mitochondrial membrane permeability in HeLa cell line. Human cervical cancer HeLa cell lines were treated with daidzin and doxorubicin for 24 h. The cells stained with Rhodamine 123 stain and representative stained cells of each group were depicted. Control (A), daidzin treated cells (B) and doxorubicin treated cells (C). The decreased green fluorescence (yellow arrows) in the daidzin and Dox treated cells proved the depleted MMP levels in the HeLa cells.
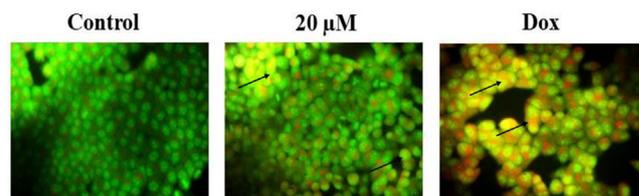

Fig 4. Daidzin induces apoptosis in HeLa cell line. Human cervical cancer HeLa cell lines were treated with daidzin and doxorubicin for 24 h. The cells stained with dual stain acridine orange and ethidium bromide (1:1). The representative stained cells of each group were depicted. Control (A), daidzin treated cells (B) and doxorubicin treated cells (C). The higher number of red and orange colored cells (black arrows) were demonstrated the higher amount of apoptotic cell death in the HeLa cells.

daidzin treated cells increased number shown increased number of green-fluorescence cells indicating early apoptotic cells (B). Increased number of orange fluorescence emitting late apoptotic cells were observed in doxorubicin treated cells (C).

4.2. Daidzin impairs cell adhesion property of HeLa cell line

Fig. 5 depicts the results of matrigel gel cell adhesion assay of HeLa cell line treated with daidzin and doxorubicin. Control untreated group cells shown increased adherent cells compared to the daidzin and doxorubicin treated cells. Daidzin treatment decreased the adherent cells number (B) which comparatively equal to the number of cells observed in anticancer drug doxorubicin treated group (C).

4.3. Daidzin increases caspases activity in HeLa cell line

Caspases, cysteine proteases are the executors of programmed cell death apoptosis, hence we assessed Caspase 8 and Caspase 9 levels in daidzin treated HeLa cell line. Compared to control

Fig 1. Daidzin induces cytotoxicity in HeLA cell line. Human cervical cancer HeLa cell lines were treated with different concentrations of daidzin (2.5–100 μM) for 24 h. The cells were subjected to MTT assay and the values depicted as ± SD of three individual experiments. Daidzin treatment effectively reduced the viability of HeLa cells in a dose dependent manner.
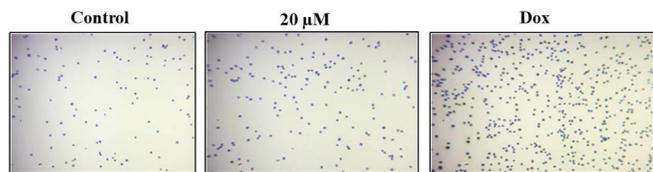
Fig 5. Daidzin impairs cell adhesion property of HeLa cell line. Human cervical cancer HeLa cell lines were treated with daidzin and doxorubicin for 24 h. The control and treated cells were subjected to matrigel cell adhesion assay. The representative stained cells of each group were depicted. Control (A), daidzin treated cells (B) and doxorubicin treated cells (C).

untreated cells daidzin treated cells shown increased level of caspase 8 and caspase 9 activity. Significantly doxorubicin treated cells shown increased levels of caspase activity compared to daidzin treated cells (Fig. 6).

4.4. Daidzin inhibits inflammation in HeLa cell line

Fig. 7 signifies the results of qPCR analysis of inflammatory gene expression in daidzin treated HeLa cells. Doxorubicin treated cells shown significant decrease in the gene expression TNF-α, NFκB, IL-6, COX-2 than the control and daidzin treated cells. Compared to untreated cells daidzin treated cells shown significant decreased in inflammatory gene expression (Fig. 7).

4.5. Daidzin inhibits proliferation in HeLa cell line

JAK/STAT is a cornerstone for cancer cell progression and it's a key modulator of immune surveillance. Daidzin treatment significantly decreased the gene of expression JAK2, STAT3 and ERK compared to the control untreated cells. Drastic decrease in JAK2, STAT3 and ERK gene expression were observed in the cells treated with doxorubicin (Fig. 8).

5. Discussion

One of the most threatening health concern of global population is cancer, cancer related mortalities accounts to be the second leading cause of death (Huang et al., 2010). By the year 2050, it has been predicted about 27 million people will be affected with cancer and the annual mortality is to be approximately 17.5 million. Dietary factors play a critical role in cancer induction, 30–35% of cancer cases were reported to be associated with diet (Mazzocchi et al., 2019). Everyday consumption of nutrients rich food have reduced the incidence rate of cancer induction (Shaikh et al., 2019). Phytochemicals were proven to possess antioxidant, anti-
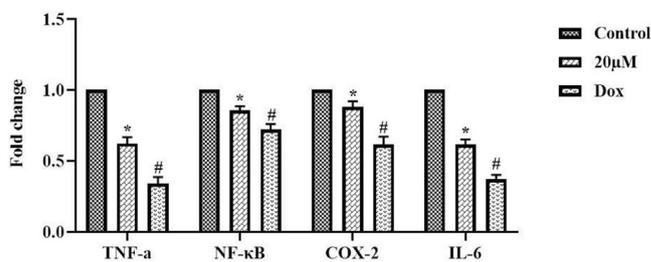

Fig 7. Daidzin inhibits inflammation in HeLa cell line. Human cervical cancer HeLa cell lines were treated with daidzin and doxorubicin for 24 h. The gene expression of proinflammatory protein TNF-α, NFκB, IL-6, COX-2 were analyzed with qPCR and the results were expressed as fold change. The experiments were performed in triplicates. p < 0.05 was considered as statistical difference and the results were expressed as mean ± SD.
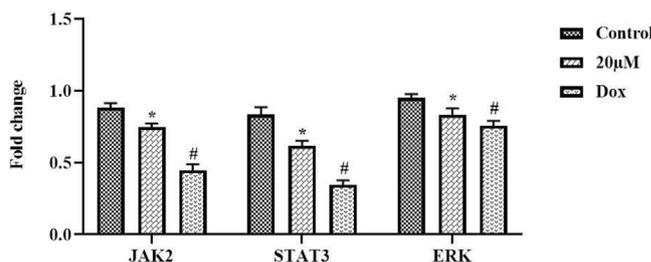

Fig 8. Daidzin inhibits proliferation in HeLa cell line. Human cervical cancer HeLa cell lines were treated with daidzin and doxorubicin for 24 h. The gene expression of cell proliferating proteins JAK2, STAT3 and ERK were analyzed with qPCR and the results were expressed as fold change. The experiments were performed in triplicates. p < 0.05 was considered as statistical difference and the results were expressed as mean ± SD.

carcinogenic, antiviral, antiallergic, anti-inflammatory properties (Huang et al., 2010). Daidzin is one such phytochemical belonging to polyphenol group possess numerous pharmacological properties.

In current study the anticancer activity of daidzin against human cervical cancer cell was assessed in vitro with HeLa cell line. Our MTT results shown, daidzin significantly reduced the cell viability of HeLa cell line which correlates with previous studies where daidzin displayed antiproliferative effect in prostate cell line (Rabiau et al., 2010), breast cancer (Liu et al., 2012b), hepatocarcinoma SK-HEP-1 cells (Park et al., 2013a). 20 μM daidzin exhibited 50% of cell death hence for the further studies we used 20 μM concentration of daidzin.

Reactive oxygen species are double edged swords which promote and inhibit the cancer growth. ROS alters the tumor signaling
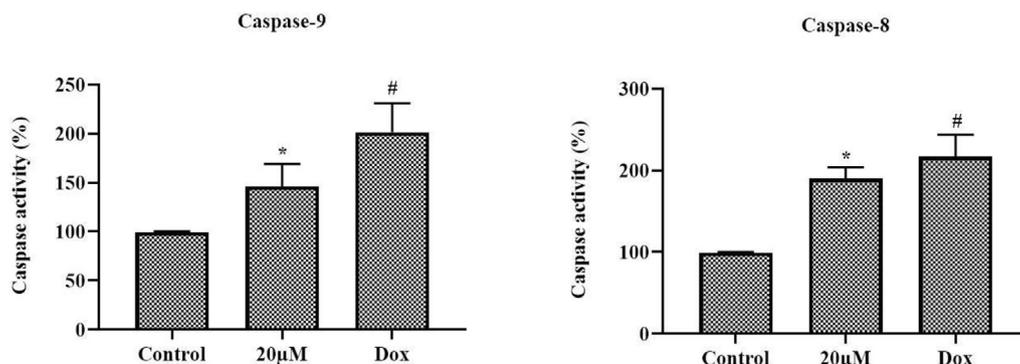

Fig 6. Daidzin increases caspases activity in HeLa cell line. Human cervical cancer HeLa cell lines were treated with daidzin and doxorubicin for 24 h. Cell lysates were subjected to estimation of caspase activity using ELISA kit. The values were depicted as ± SD of three individual experiments.

pathways and promotes cancer cell proliferation, invasion, angiogenesis (Sabharwal & Schumacker 2014). It also causes oxidative stress and inhibits the tumorigenic signaling pathway (Nogueira et al. 2008). Increased ROS levels converts cancer cells more vulnerable to oxidative stress induced cancer cell death. Therefore elevating the levels of reactive oxygen species and impairing the production of antioxidants in the cancer cells may be effective treatment to for cancer. Ichijo et al. (1997) reported the increased ROS levels promoted cell death in cancer via activation of ASK1/JNK and ASK1/p38 signaling pathway. In our study daidzin increased the generation of ROS in HeLA cells which would have induced apoptosis.

Apoptosis, is one of the effective tool to inhibit cancer cell progression. Anticancer drugs targeting apoptotic signaling proteins are effectively inhibits the cancer cells (Wang & Youle, 2009). Hence the apoptotic property of daidzin against HeLa cells was examined. Mitochondria play a key role in apoptosis induction, it maintains the ATP production and the mitochondrial membrane potential regulates the release of apoptogenic factor from intermembrane space into the cytosol during apoptosis signaling (Singh et al., 2019). Daidzin impaired the mitochondrial membrane in HeLa which is evidenced in our Rhodamine 123 staining results. Our results of AO/EtBr staining shown increased number of apoptotic cells which confirms daidzin increased the ROS generation altered the mitochondrial membrane potential thereby induced apoptosis.

Caspases are cysteine proteases which plays a key role in apoptosis via cleaving the target proteins (Zaman et al., 2014). Caspases are classified into initiator caspase and executor caspases, in the current study daidzin treatment increased the levels of both intrinsic and extrinsic initiator caspases of apoptotic pathway. The increase in caspases would have initiated apoptosis in HeLa cells which is evidenced with our staining results.

NFκB, is a key regulator of proteins involved in cell proliferation, inflammation and apoptosis. NFκB regulates the expression proinflammatory cytokines TNF-α, IL-6, COX-2 which are involved in cancer cell progression. The over expression of these cytokines was reported in various cancer cells (Betzler et al., 2020). In the present daidzin significantly decreased the expression of NFκB which in turn down regulated the proinflammatory cytokine expression. Daidzin also decreased the expression of cell proliferating protein JAK2, STAT3 and ERK expression which may be the reason for decreased number of adherent cells reported in cell adhesion. Daidzin effectively inhibited cell growth and induced apoptosis in human cervical cancer HeLa cell line.

6. Conclusion

To conclude, daidzin, a polyphenolic compound effectively induced apoptosis in human cervical cancer HeLa cell line. It increased the ROS generation, altered the mitochondrial membrane permeability and inhibited the cell adhesion property of HeLa cells. Daidzin decreased expression of proinflammatory cytokine protein and cell proliferating proteins. Over all our results confirms anticancer role of daidzin and the additional studied on this framework could leads to the development of potent anticancer drug to treat cervical cancer in the future, as an alternative for current allopathic drugs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further Reading

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