Tropical Journal of Pharmaceutical Research January 2022; 21 (1): 79-85
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i1.13

Original Research Article

Effect of fisetin on the proliferation and migration of human breast and cervical cancer cells

Benjaporn Buranrat[1]*, Mutita Junking[2]
[1]Faculty of Medicine, Mahasarakham University, Muang District, Maha Sarakham 44000, [2]Division of Molecular Medicine, Research Department, Faculty of Medicine, Siriraj Hospital Mahidol University, Bangkok 10700, Thailand

For correspondence: **Email: buranrat@gmail.com*

Sent for review: 13 February 2021 *Revised accepted: 6 January 2022*

Abstract

Purpose: *To explore the fisetin effects on the growth, apoptosis, and migration in human breast and cervical cancer cells, MCF-7 and HeLa cells, respectively.*
Methods: *Cell cycle arrest was used for the determination of cell growth and sulforhodamine B (SRB) colony formation. Gene expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR), while cell migration was assessed by wound healing and matrigel migration assays.*
Results: *The data indicate that fisetin activated breast and cervical cancer cell death, and this was confirmed by decreased cell growth, changed cell morphology, and arrested cell cycle at G2/M phase. During the incubation period, fisetin inhibited cancer cells at low concentration with half-maximal concentration (IC_{50}) values of 267.10 ± 48.96, 88.42 ± 1.35 and 41.03 ± 8.04 µg/mL after 24, 48 and 72 h, respectively, for MCF-7 cells; and 140.97 ± 22.92, 100.84 ± 10.97 and 95.53 ± 14.33 µg/mL, respectively, for HeLa cells ($p < 0.05$). Fisetin suppressed the migration of the cancer cells by inhibiting wound healing and suppressing cell migration to the lower chamber. Moreover, fisetin inhibited cancer cells growth by reducing the gene expression of cyclin D1 and cyclin E.*
Conclusion: *Fisetin reduces cancer cell death, activates apoptosis and suppresses cancer cell migration. Therefore, fisetin is a potential anticancer agent for the prevention and treatment of breast and cervical cancers in humans, but further development studies are required in this regard.*

Keywords: *Fisetin, Breast cancer, Cervical cancer, Cell death, Cell migration*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Two types of cancer namely, breast and cervical cancer, are said to be the most prevalent cancer types affecting women worldwide. Presently, breast cancer is associated with a higher incidence and mortality rate among cancers that predominantly affect females [1]. Consequently, there have been developments in the management of human breast cancer, particularly in hormone-targeted and biological agents-targeted therapy [2]. Nevertheless, these therapies may only benefit certain patients, and others may have rapid progression and high toxicity, but the response may still be poor [3]. Novel agents/compounds are urgently required in the development process of new strategies against breast cancer. Additionally, cervical

cancer is the second female cancer with high incidence in developing countries [4] including Thailand. The standard treatment for this cancer include chemotherapy and surgery. Presently, herbal medicine, natural food or traditional agents are being used for the treatment for various cancers to improve or replace the standard treatment regimens that have high toxicities. This study therefore seeks to explore the effects of the new compounds against many cancers cell types, with fisetin being the compound of interest [5].

Fisetin is a natural flavonoid that is primarily present in vegetables and fruits such as apples, persimmon, strawberries, cucumber, and onions [6]. It is known to possess multiple pharmacological attributes, including antioxidative, anti-inflammatory, anti-angiogenesis, apoptosis, and anti-growth effects. Fisetin has been shown to be toxic to several cancer types such as liver [20], leukemia [21], lung [22], breast, cervical, and prostate cancers [23]. Fisetin inhibits their growth and stimulates apoptosis in various cancer cells via several mechanisms. It similarly inhibits colon cancer cell growth through the reduction of COX-2 and Wnt-EGFR-NF-κB [24], then stimulates caspase 3 signaling pathway in liver cancer cells [20], and also activates caspase 3 activity and $Ca2^{+}$-dependent endonuclease in leukemic cells [11]. Previously, fisetin also induces autophagic death in prostate cancer cells via the inhibition of mTORC1 and mTORC2 pathways [26]. However, fisetin effects on female cancer cells have not been well documented. Based on the data obtained, this work investigated the inhibitory actions of fisetin on two female cancer cells, human MCF-7 breast and HeLa cervical cancer cells, by determining their growth and migration and exploring the underlying molecular mechanisms of action.

EXPERIMENTAL

Cell lines and cell cultures

Human MCF-7 breast and cervical HeLa cancer cells lines were obtained from the ATCC. The two cell lines were cultured in 10 % FBS in DMEM with streptomycin and penicillin.

Sulforhodamine B (SRB) and morphology assay

SRB method was designed to detect cancer cell death. The cancer cells were plated in a 96-well plate ($1x10^4$ cells/well) and the next day, the cells were exposed to different concentration of fisetin (0 - 250 mM). After various incubation periods, cell death was measured at 24 - 72 h by SRB assay. The cancer cells were fixed, stained with 0.4% SRB dye, and solubilized. The absorbance was examined measured at 540 nm with a spectrophotometer. Cell morphology was performed to detect the death of the cancer cells. The cancer cells were cultured in culture plates ($1x10^4$ cells/well) overnight, and then incubated with fisetin (0 - 250 μM). After culturing another for 24 h, morphological changes were detected in the cancer cells and captured using inverted microscopy (Olympus, magnification, *20x*).

Colony formation assay

Colony formation was made to detect the ability of cancer cell to form colonies. The two cancer cell lines were cultured in culture plates (500 cells/well) overnight at 37˚C and then incubated with fisetin (0 - 250 μM) for 24 h. Next, the cells were cultured further for another 15 days with complete DMEM medium, and then the cell colonies were fixed and stained with 0.5% crystal violet.

Cell cycle distribution assay

Cell cycle distributions were performed to detect the MCF-7 and HeLa cancer cell proliferation. The cancer cells were cultured into a culture plate ($2.5x10^5$ cells/well) overnight and then exposed to different concentrations of fisetin (0 - 250 mM) for 24 h. Subsequently, the cells were fixed, and 1000 μL PI solution (Cat No. 550825, BD Biosciences, CA, USA) was added to the cell pellet at 4°C for 30 min in the dark. The percentage of cell cycle distributions was measured by flow cytometer (BD Biosciences, CA, USA) using BD Accuri C6 Plus software, and the fluorescent signals were displayed as histograms.

Wound healing assay

The wound healing method was used to explore the capability of the two cancer cells to migrate. The cells were cultured in 24-well plates ($2.5x10^5$ cells/well), and were allowed to reach ~80% confluence, and then a wound was made on them using 0.2 mL pipette tips, and exposed to different concentrations of fisetin (0 - 250 mM) for 48 h. The wound was photographed at 0 and 48 h to examine the wound by measuring the denuded area.

Matrigel migration assay

Cell migratory abilities were evaluated using Transwell assay. The cancer cells ($2x10^4$ cells/ml in 0% FBS DMEM) were plated in the upper

chamber with fisetin (0 - 250 mM), and in the lower chamber, 10% FBS medium was added and then incubated for 24 h at 37˚C. Afterwards, the migrated-cells were then fixed and stained with 0.5% crystal violet. The migrated cells were photographed, measured and counted with inverted microscope (CKX53, Olympus Corporation, *20x* magnification).

Gene expression assay

qRT-PCR was designed to examine *cyclin D1* and *cyclin E* mRNA expression. Cancer cells were plated in cultured plate ($2.5x10^5$ cells/well) overnight and then exposed to fisetin (0 - 250 mM) for 24 h. Following treatment, the cells were lysed, and total RNA was extracted and converted to cDNA. The 20 mL of reaction medium was composed of PCR mixture and primers. The primer sequences are shown in Table 1. The mRNA levels were calculated using the $2^{-\Delta\Delta Cq}$ analysis, and standardized to the internal reference genes.

Table 1: Primer sequences for cyclin D1, cyclin E, and beta-actin

Gene	Primer sequence	
Cyclin D1	Forward	5' GCTGCGAAGTGGAAACCATC 3'
	Reverse	5'CCTCCTTCTGCACACATTTGAA3'
Cyclin E	Forward	5'ACTCAACGTGCAAGCCTCG3'
	Reverse	5'GCTCAAGAAAGTGCTGATCCC 3'
Beta-actin	Forward	5'GTGACGTTGACATCCGTAAAGA-3'
	Reverse	5' -GCCGGACTCATCGTACTCC-3'

Statistical analysis

The differences between un-treated and treated groups were determined by Student's *t-test*. Results are presented as the mean ± SEM and statistical analysis was operated using GraphPad Prism 5.0 (GraphPad Software, Inc). $P < 0.05$ was taken as statistically significant.

RESULTS

Effect of fisetin on cell morphology and cell death

The inverted microscope was used to examine MCF-7 and HeLa cancer cell morphology alteration after exposure to fisetin for 24 h. From the results, fisetin inhibited cell viability in a concentration-dependent manner in the two cancer cell lines (Figure 1 A and C). When the cells were exposed to various concentrations of fisetin, they indicated morphology changes as well as apoptotic features such as apoptotic body formation, cellular shrinkage, oval or irregular shape, and condensed cytoplasm.

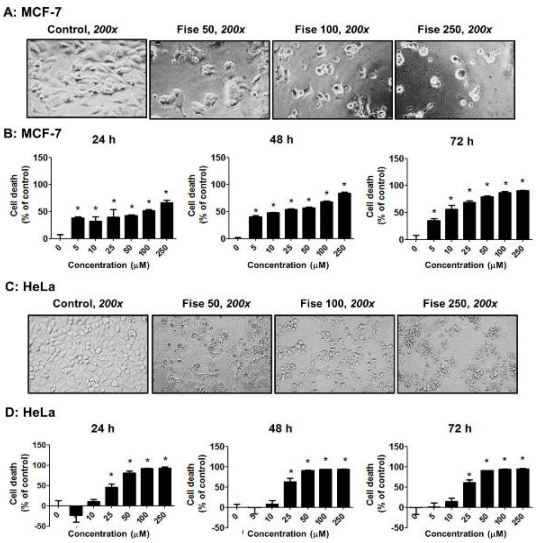

Figure 1: Effect of fisetin on cell morphology and cell death in MCF-7 and HeLa. (A and C) Morphology of the cancer cells were investigated after treating with fisetin for 24 h under inverted phase contrast microscopy (magnification, *x20*). (B and D) The cancer cell death was examined by SRB assay after exposing the two cancer cell lines to fisetin for 24, 48 and 72 h respectively. *$P < 0.05$

To explore fisetin effects on the death of the cancer cells. Fisetin inhibited the viability of the two cancer cells in a concentrated and time-dependent manner (Figure 1 B and D). In addition, it was discovered that cell death could be significantly increased with an incubation period starting at 24 h at 5 to 250 mM for MCF-7 cells, and 25 to 250 mM for HeLa cells. The IC_{50} values of MCF-7 cells was 267.10 ± 48.96, 88.42 ± 1.35, 41.03 ± 8.04 mM for 24, 48, and 72 h, respectively. Furthermore, IC_{50} values of HeLa cells were 140.97 ± 22.92, 100.84 ± 10.97, 95.53 ± 14.33 mM for 24, 48, and 72 h respectively (Table 2). Fisetin suppressed the proliferation of both breast and cervical cancer cell lines, and the IC_{50} were the same; however, MCF-7 cells may be more sensitive with fisetin than HeLa cells at 48 and 72 h incubation periods.

Effect of fisetin on cell cycle progression and colony formation

To confirm the effect of fisetin on cell proliferation, the cell cycle progression and colony formation were determined. The effect of

fisetin on cell cycle profile was measured using flow cytometric analysis. For the MCF-7 cells, the percentage of cells in G0/G1 phase increased especially at 100 mM of fisetin (Fig. 2A), and decreased at 250 mM. For the HeLa cells, the percentage of cells in G2/M phase increased at 100 mM; however, it was not statistically significant when compared with the un-treated cells (Fig. 2B). With a fisetin dosage of 250 mM, G0/G1 phase reduced the height of the histogram when compared with the control group.

Table 2: IC_{50} and Emax of fisetin on breast and cervical cancer cells

Cell line	Incubation time (h)	IC_{50} (mg/mL)	Emax
MCF-7	24	267.10±48.96	66.49±4.63
	48	88.42±1.35*	83.58±2.24*
	72	41.03±8.04*	90.36±0.93*
HeLa	24	140.97±22.92	92.98±3.09
	48	100.84±10.97*	93.42±1.55
	72	95.53±14.33*	94.41±0.82

*Significant when compared with 24 h incubation period of each group

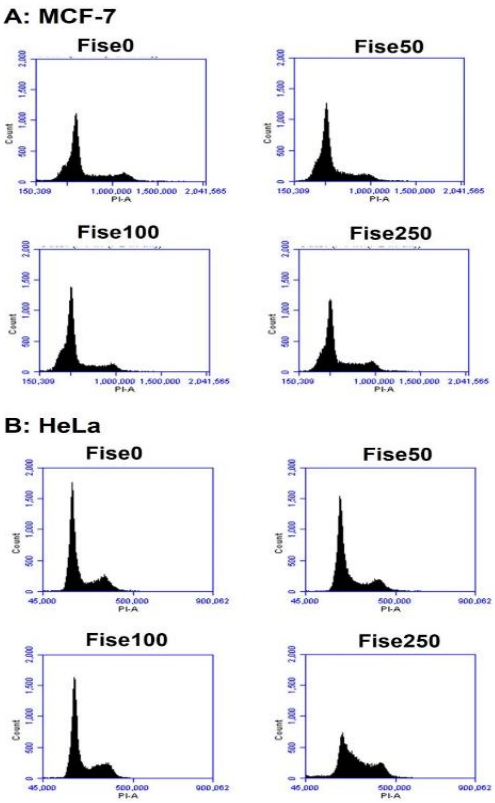

Figure 2: Effect of fisetin on cell cycle arrest in cancer cells. (A and B) Cell cycle arrest was determined after the two cancer cell lines were to be exposed with fisetin for 24 h and then analyzed by flow cytometry

Subsequently, a colony formation method was used to analyze the proliferative viability (Figure 3 A and B) of the two cancer cell lines. Fisetin suppressed the colony formation ability and significance at the dose of 50, 100, 250 mM after incubating for 24 h and cultured further for 15 days to form the colonies.

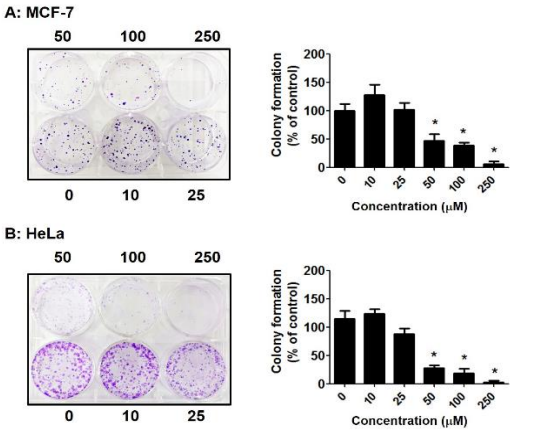

Figure 3: Effect of fisetin on colony formation in cancer cells. (A and B) The colony formation of the cancer cell lines were examined after exposure to fisetin for 24 h and then cultured for a further 15 days. *$P < 0.05$

Cell migration

The role of fisetin on cell migration was also explored using wound healing and matrigel migration method. The wound healing results indicate that fisetin inhibited cell migration after exposure to nfisetin at 48 h in MCF-7, and at 24 h in HeLa cells; furthermore, the inhibition was significant at doses of 50, 100, 250 mM for both cancer cells ($p < 0.05$, Figure 4 A and B).

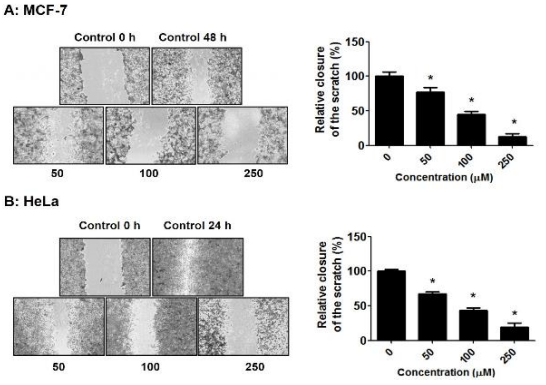

Figure 4: The fisetin effects on migration. (A and B). The two cancer cells were treated with fisetin for 48 h (MCF-7 cells) and 24 h (HeLa cells), and the denuded area was measured to calculate the migration assay. *$P < 0.05$

The migration ability was suppressed after observation using the Transwell assay, which is similar to the wound healing method (Fig. 5 A and B), and the number of migrating cells were significantly decreased, and displayed the highest activity at 50 - 250 mM in both cancer cells.

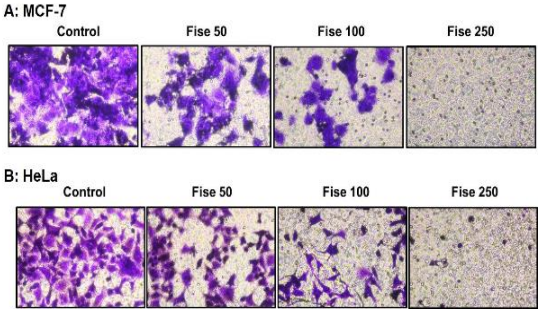

Figure 5: The effects of fisetin on migration in cancer cells. (A and B). The migration assay was examined by matrigel migration assay after treatment with fisetin for 24 h

Effect of fisetin on gene-related cell proliferation

To examine the fisetin effects on gene-related cell proliferation expression, qRT-PCR assay was used. Fisetin, at 250 mM, caused reduction of *cyclin D1* mRNA expression in both cancer cell lines. Interestingly, fisetin suppressed *cyclin E* on HeLa cells and showed a slight effect in MCF-7 cells; (Figure 6 A and B). Hence, fisetin inhibited cell proliferation by decreasing gene-related cell proliferation, cyclin D1 and *cyline E* in human breast and cervical cancer cells.

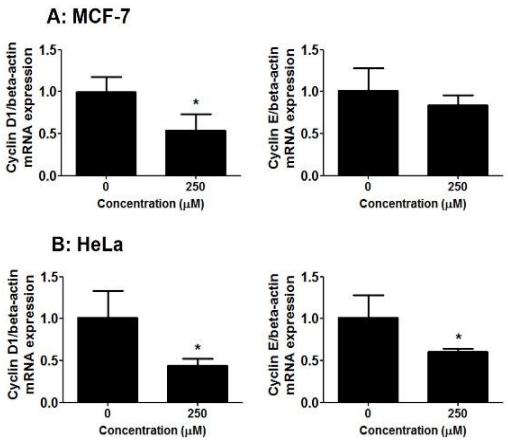

Figure 6: The effects of fisetin on *cyclin D1* and *cyclin* E gene expression levels. (A and B). The two cancer cells were exposed with 250 mM fisetin for 24 h and then measured the *cyclin D1* and *cyclin E* mRNA expression by RT-PCR. **P* < 0.05

DISCUSSION

Flavonoid compounds are broadly studied in traditional medicine, especially fisetin. Previous studies found that fisetin suppressed the human breast cancer cell proliferation by activating apoptosis and autophagy [7], and inhibited the cervical cancer cells growth via the activation of ERK1/2 and caspase 3/8 levels [8]. However, fisetin *in vitro* studies which compare the cell proliferation and migration attributes of the breast and cervical cancer cells are still unclear. This study indicates that fisetin suppressed the cancer cell growth and migration of the human breast cancer MCF-7 cells, similar to cervical cancer HeLa cells with high activity. Fisetin decreased gene-related cell proliferation and *cyclin D1* gene expression levels in both cancer cells. However, *cyclin E* was suppressed only in HeLa cells. The results also showed that fisetin suppressed breast and cervical cancer cells growth and migration by inhibiting *cyclin D1* gene expression levels.

Based on the present study, it was determined that fisetin had the same effect on cell growth and migration in breast and cervical cell lines. Next, the effect of fisetin on the two cancer cell types was discovered. IC_{50} values in this work were at similar levels on MCF-7 and HeLa cells, similar to the other cancer cells such as in the prostate [9], bladder [10], liver [11] and colon [12], and it had a more sensitive effect on cancer cells than normal cells [8]. Fisetin also showed good anti-cancer effects, and showed the less adverse effects on the normal cells. Conversely, on cancer cells, fisetin had more efficacy and broad-spectrum ability to defeat them using a different mechanism which imposes less harm on normal cells.

From the data obtained, we can see that fisetin inhibited cancer cell proliferation and induced the death of cancer cells by utilizing several mechanisms. It induces mitochondrial dysfunction by generating ROS levels, leading to gastric cancer cell apoptosis [13]. In addition, fisetin stimulated the mTOR repressor via the suppression of Akt and stimulation of AMPK, leading to proliferative suppression [12]. Moreover, fisetin inhibited protein-controlled cell growth by reducing the CDK-2 and CDK-4 that activated cyclin E and D1 reduction in colon cancer cells [14]. Following cell cycle arrest, it inhibited the cell cycle distribution from the G1 phase to S phase at 8 h, and G2/M phase arrest after a 24 h incubation period [14]. In line with our data, MCF-7 cells stopped the cell cycle at G0/G1 phase after incubating with 100 mM fisetin for 24 h, whereas HeLa cells were also

arrested at G2/M phase. Fisetin suppressed prostate cancer cells in the G1 phase that was related to decreasing the cyclin D1, D2 and E protein levels [15]. It could be concluded that fisetin had more potent effects on many cancer cell types including breast and cervical cancer.

The detailed relationship between fisetin and migration lacked detailed information. Our results indicated that fisetin inhibited two cancer cells with low concentration. From the previous data, fisetin decreased the cancer cell migration by inhibiting PAK4 pathways in human oral squamous cell carcinoma [16], and suppressed migration of cervical cancer cells by reducing uPA level via the attenuation of both the NF-κB and p38 signaling pathway [17]. With regards to human breast cancer cells, results indicated that fisetin decreased triple-negative breast cancer metastasis by inhibiting the PTEN/Akt/GSK3β pathway [18]. In combination with its anti-cancer effects, fisetin augmented the anti-invasive and anti-metastatic potential of sorafenib in melanoma thru the decrease in N-cadherin, vimentin, fibronectin, MMP-2 and MMP-9 levels [19]. It can therefore be concluded that fisetin attenuated the cancer cell migration of several cancer cell types, breast and cervical cancer included.

CONCLUSION

The study is the first report to show preliminary data on the comparative effects of fisetin on breast and cervical cancer cell types. The findings show that fisetin has similar inhibitory effects on breast and cervical cancer cell proliferation and migration. It inhibits cell growth and stops cancer cell progression at G0/G1 phase in breast cancer, and G2/M phase in cervical cancer cell. Furthermore, it suppresses cancer cell migration both in wound healing and matrigel migration assays. Thus, the mechanism of action involves the reduction of gene-related cell proliferation, including *cyclin D1* and *cyclin E*. Fisetin is useful for the prevention and treatment of breast and cervical cancer.

DECLARATIONS

Acknowledgement

This research project was financially supported by Mahasarakham University 2021.

Conflict of interest

No conflict of interest is associated with this work.

Authors' contribution

The authors declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Benjaporn Buranrat and Mutita Junking conducted the experimental model and wrote the manuscript. Benjaporn Buranrat designed the protocol and performed statistical analysis.

Open Access

REFERENCES

1. *Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.*
2. *Hu X, Huang W, Fan M. Emerging therapies for breast cancer. J Hematol Oncol 2017; 10: 98. http:// DOI 10.1186/s13045-017-0466-3.*
3. *Wang C, Kar S, Lai X, Cai W, Arfuso F, Sethi G, Lobie PE, Goh BC, Lim LHK, Hartman M, Chan CW, Lee SC, Tan SH, Kumar AP. Triple negative breast cancer in Asia: An insider's view. Cancer Treat Rev 2018; 62: 29-38.*
4. *Smith HO, Tiffany MF, Qualls CR, Key CR. The rising incidence of adenocarcinoma relative to squamous cell carcinoma of the uterine cervix in the United States--a 24-year population-based study. Gynecol Oncol 2000; 78: 97-105.*
5. *de Sousa RR, Queiroz KC, Souza AC, Gurgueira SA, Augusto AC, Miranda MA, Peppelenbosch MP, Ferreira CV, Aoyama H. Phosphoprotein levels, MAPK activities and NFkappaB expression are affected by fisetin. J Enzyme Inhib Med Chem 2007; 22: 439-444.*
6. *Moon YJ, Wang X, Morris ME. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro 2006; 20: 187-210.*
7. *Yang PM, Tseng HH, Peng CW, Chen WS, Chiu SJ. Dietary flavonoid fisetin targets caspase-3-deficient human breast cancer MCF-7 cells by induction of caspase-7-associated apoptosis and inhibition of autophagy. Int J Oncol 2012; 40: 469-478.*
8. *Ying TH, Yang SF, Tsai SJ, Hsieh SC, Huang YC, Bau DT, Hsieh YH. Fisetin induces apoptosis in human cervical cancer HeLa cells through ERK1/2-mediated*

activation of caspase-8-/caspase-3-dependent pathway. Arch Toxicol 2012; 86: 263-273.

9. Haddad AQ, Fleshner N, Nelson C, Saour B, Musquera M, Venkateswaran V, Klotz L. Antiproliferative mechanisms of the flavonoids 2,2'-dihydroxychalcone and fisetin in human prostate cancer cells. Nutr Cancer 2010; 62: 668-681.
10. Li J, Cheng Y, Qu W, Sun Y, Wang Z, Wang H, Tian B. Fisetin, a dietary flavonoid, induces cell cycle arrest and apoptosis through activation of p53 and inhibition of NF-kappa B pathways in bladder cancer cells. Basic Clin Pharmacol Toxicol 2011; 108: 84-93.
11. Chen YC, Shen SC, Lee WR, Lin HY, Ko CH, Shih CM, Yang LL. Wogonin and fisetin induction of apoptosis through activation of caspase 3 cascade and alternative expression of p21 protein in hepatocellular carcinoma cells SK-HEP-1. Arch Toxicol 2002; 76: 351-359.
12. Suh Y, Afaq F, Khan N, Johnson JJ, Khusro FH, Mukhtar H. Fisetin induces autophagic cell death through suppression of mTOR signaling pathway in prostate cancer cells. Carcinogenesis 2010; 31: 1424-1433.
13. Sabarwal A, Agarwal R, Singh RP. Fisetin inhibits cellular proliferation and induces mitochondria-dependent apoptosis in human gastric cancer cells. Mol Carcinog 2017; 56: 499-514.
14. Lu X, Jung J, Cho HJ, Lim DY, Lee HS, Chun HS, Kwon DY, Park JH. Fisetin inhibits the activities of cyclin-dependent kinases leading to cell cycle arrest in HT-29 human colon cancer cells. J Nutr 2005; 135: 2884-2890.
15. Khan N, Afaq F, Syed DN, Mukhtar H. Fisetin, a novel dietary flavonoid, causes apoptosis and cell cycle arrest in human prostate cancer LNCaP cells. Carcinogenesis 2008; 29: 1049-1056.
16. Li Y, Jia S, Dai W. Fisetin Modulates Human Oral Squamous Cell Carcinoma Proliferation by Blocking PAK4 Signaling Pathways. Drug Des Devel Ther 2020; 14: 773-782.
17. Chou RH, Hsieh SC, Yu YL, Huang MH, Huang YC, Hsieh YH, Fisetin inhibits migration and invasion of human cervical cancer cells by down-regulating urokinase plasminogen activator expression through suppressing the p38 MAPK-dependent NF-kappaB signaling pathway. PLoS One 2013; 8: e71983. http:// doi: 10.1371/journal.pone.0071983.
18. Li J, Gong X, Jiang R, Lin D, Zhou T, Zhang A, Li H, Zhang X, Wan J, Kuang G, Li H. Fisetin Inhibited Growth and Metastasis of Triple-Negative Breast Cancer by Reversing Epithelial-to-Mesenchymal Transition via PTEN/Akt/GSK3beta Signal Pathway. Front Pharmacol 2018; 9: 772. http:// doi: 10.3389/fphar.2018.00772.
19. Pal HC, Diamond AC, Strickland LR, Kappes JC, Katiyar SK, Elmets CA, Athar M, Afaq F. Fisetin, a dietary flavonoid, augments the anti-invasive and anti-metastatic potential of sorafenib in melanoma. Oncotarget 2016;7: 1227-1241.