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ORIGINAL ARTICLE

Phloretin flavonoid exhibits selective antiproliferative activity in doxorubicin-resistant gastric cancer cells by inducing autophagy, inhibiting cell migration and invasion, cell cycle arrest and targeting ERK1/2 MAP pathway

Qing You*, JiaPeng Xu*, ZhenXin Zhu, Zunqi Hu, QingPing Cai

Department of Gastrointestinal Surgery, Changzheng Hospital, Second Military Medical University, Shanghai, 200003, China.

*These two authors contributed equally to this work.

Summary

Purpose: *Studies have shown that Phloretin exerts anticancer effects on several types of cancer cells. Nonetheless, the anticancer effects of Phloretin have not been fully explored against the human gastric cancer cells. Therefore, this study was undertaken to evaluate the anticancer effects of Phloretin against the human gastric cancer cells.*

Methods: *Cell proliferation was evaluated by WST-1 assay while cell cycle analysis was carried out by flow cytometry. The effects on cell migration and invasion were evaluated by wound healing assay and transwell assays, respectively. Electron microscopy and western blot methods were used to study effects on autophagy and ERK1/2/MAPK signalling pathway.*

Results: *The results showed that Phloretin inhibited the proliferation rate of the human SNU-1 gastric cancer cells and showed an IC_{50} of 18 μM. However, Phloretin showed very high IC_{50} (80 μM) against the normal GES-1 normal gastric cells. Electron microscopy showed that Phloretin triggered autophagy in the SNU-1 gastric cancer cells which was accompanied by enhancement in the expression of LC3B II and Beclin 1. Cell cycle analysis showed that Phloretin caused accumulation of the SNU-1 cells in the G0/G1 phase of the cell cycle triggering G0/G1 cell cycle arrest. The G0/G1 arrest of SNU-1 cells was also associated with depletion of cyclin D1 and D2 expression. Wound healing and transwell assays showed that Phloretin suppressed the migration of the SNU-1 gastric cancer cells, suggestive of the anti-metastatic potential of this molecule. Finally, this molecule also blocked the ERK1/2/MAPK signalling pathway in SNU-1 cells in a concentration-dependent manner.*

Conclusions: *Phloretin may prove beneficial as a promising drug candidate for gastric cancer treatment provided further studies are carried out on it, especially toxicological studies.*

Key words: *gastric cancer, phloretin, autophagy, cell migration, cell cycle arrest*

Introduction

Gastric cancer is one of the lethal malignancies causing tremendous human mortality. Approximately 0.65 million deaths and 0.9 million new cases of gastric cancer were detected in 2002 alone [1]. Gastric cancer shows geographic variation, but the developing countries have shown higher incidence of this disease. The 5-year survival rates have improved for gastric cancer but they are still less than that of other cancer types [2]. Lack of efficient chemotherapeutic drugs without side effects imposes obstacles in gastric cancer treatment [3]. Therefore, there is an urgent need for the development of new drugs to decrease the incidence of gastric cancer. Plants are highly so-

Corresponding author: QingPing Cai, MD. Department of Gastrointestinal Surgery, Changzheng Hospital, Second Military Medical University, no.415 Fengyang Rd, Shanghai, 200003, China.
Tel/Fax: +86 21 8188 6999, Email: CarmenSheppardrjb@yahoo.com, caiqingping@smmu.edu.cn
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phisticated natural chemical factories and exhibit a remarkable potential to synthesize wide array of chemical entities [4]. This study was undertaken to examine the anticancer effects of Phloretin against human gastric cancer cells and also to ascertain its molecular mechanisms.

Phloretin is a plant-derived metabolite with enormous pharmacological potential [5]. This molecule causes significant decrease in the growth of lung cancer cells [6]. The growth of leukemia cells has also been shown to be suppressed by Phloretin via induction of apoptotic cell death [7]. Another study has shown that the proliferation of liver cancer cells is suppressed by Phloretin via inhibition of type II glucose transporter [8]. Additionally, Phloretin has been reported to enhance the sensitivity of lung cancer cells to cisplatin. Nonetheless, there is hardly any study that reports the anticancer activity of Phloretin in gastric cancer [9]. Cell migration and invasion is the first step that enables the cancer cells to move to other body parts and create metastasis [10]. Herein we investigated the effects of Phloretin on the migration and invasion of gastric cancer cells. ERK1/2 MAPK signal transduction has been shown to exhibit therapeutic implications in the treatment of gastric cancer [11] and herein we also studied the effects of Phloretin on this pathway.

Methods

Cell proliferation assay

The proliferation rate of the human SNU-1 gastric cancer cells and normal GES-1 cells was monitored by WST-1 assay. In brief, SNU-1 cells were cultured in 96-well plates at the density of 2×10^5 cells/well and treated with 0 to 200 μM concentrations of Phloretin for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for 4 h. The absorbance was then measured at 450 nm using a victor 3 microplate reader to determine the proliferation.

Cell cycle analysis

The SNU-1 cells treated with 0.25% trypsin were centrifuged at 1000 r/min for 5 min in Dulbecco's Modified Eagles'Medium (DMEM, Invitrogen Life Technologies, Mass, USA). The cells were washed with phosphate-buffered saline (PBS), then the supernatant was discarded and the cells were collected simultaneously. They were washed twice with ice-cold PBS, and fixed in 80% ethanol at 4°C overnight. After washing three times with PBS, the cells were suspended in 0.1 mg/ml Annexin V/Propidium Iodide (PI) at 37°C for half an hour in the dark. Cell cycle was detected by flow cytometry and expressed as the percentage of cells in each phase of the cell cycle.

Electron microscopy

The induction of autophagy in Phloretin-treated gastric cancer cells was assessed by electron microscopy. In brief, the gastric cancer SNU-1 cells were treated with 0, 9, 18 and 36 μM Phloretin for 24 h. The cells were collected by trypsinization and washed with PBS, which was followed by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by the treatment of the cells with ethanol and embedding in resin. Thin sections were then cut with an ultramicrotome and subjected to electron microscopy.

Western blot analysis

The SNU-1 were then lysed in lysis buffer containing the protease inhibitor. Around 45 μg of proteins from each sample were subjected to separation on 10% SDS-PAGE and which was followed by transferring to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the signal was detected by Odyssey

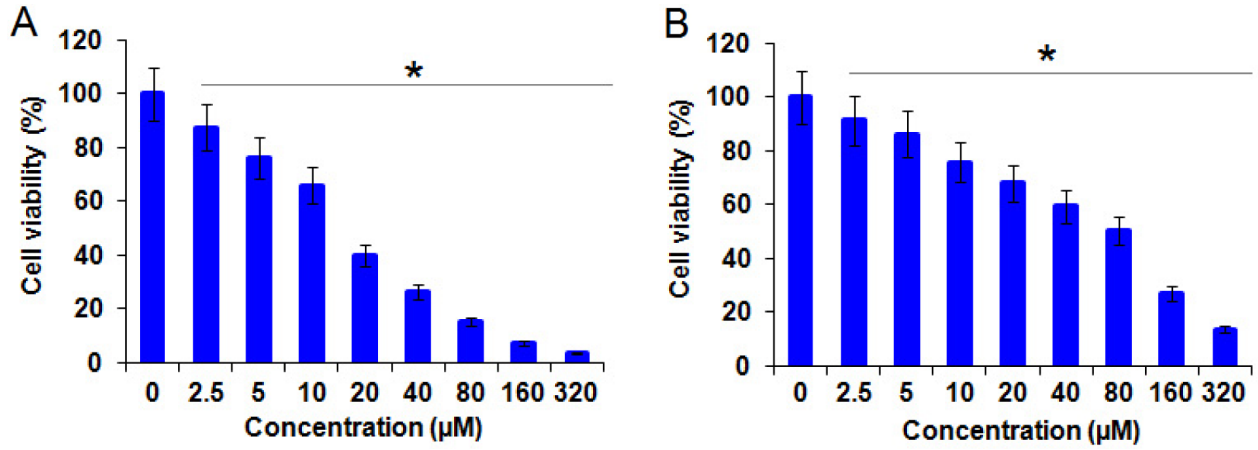

Figure 1. Effect of Phloretin on the viability of **A:** SNU-1 gastric cancer and **B:** normal GES-1 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Infrared Imaging System. Actin was used as control for normalisation.

Wound-healing assay

After treatment of the SNU-1 cells with Phloretin, DMEM was removed and the cells were washed with PBS. A sterile pipette tip was employed to scratch a wound in each well and the cells were washed again and a picture was taken. The plates were cultured at 24 h and a picture was taken again under an inverted microscope (Leica, Germany).

Cell invasion assay

The effects of Phloretin on the invasion ability of SNU-1 cells were determined by transwell chambers assay with Matrigel. Around 200 ml of cell culture were placed onto the upper chambers and only DMEM was placed in the lower chambers. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200× magnification.

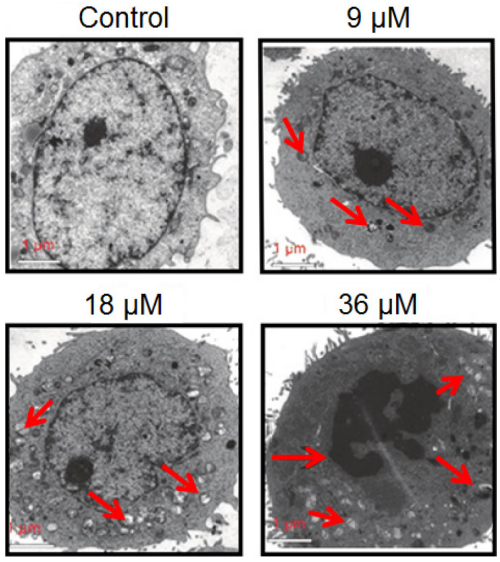

Figure 2. TEM analysis showing induction of autophagy in SNU-1 cells at indicated concentrations of Phloretin (Arrows depict autophagosomes). The experiments were performed in triplicate.

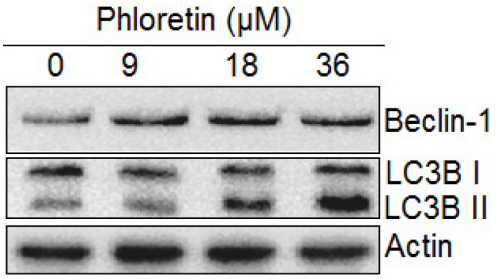

Figure 3. Effect of Phloretin on the autophagy-related proteins as depicted by western blot analysis. The experiments were performed in triplicate.

Statistics

The experiments were done in triplicate. The values presented are mean of three repeats ± SD. *p<0.05, **p<0.01 and **p<0.001 were considered statistically significant. Student's *t*-test using GraphPad prism 7 software was employed for statistical analyses.

Results

Phloretin inhibits the growth of gastric cancer cells

The WST-1 assay was used to ascertain the effects of Phloretin on the growth of the SNU-1 gastric cancer and GES-1 normal gastric cells. Phloretin caused a significant depletion in the proliferation rate of the SNU-1 cells. The effects of Phloretin on the proliferation rate of the SNU-1 cells were concentration-dependent and IC_{50} of 9 μM was reported for Phloretin against the SNU-1cells (Figure 1A). Interestingly, the effects of Phloretin on the normal GES-1 normal gastric cells were less and an IC_{50} of 80 μM was reported for Phloretin against these normal cells (Figure 1A).

Autophagy-inducing effects of Phloretin on the gastric cancer cells

Next, electron microscopic analysis of the Phloretin-treated SNU-1 cells was performed. It was observed that Phloretin caused development of autophagic vesicles or autophagosomes in the SNU-1 cells which are the hallmarks of autophagy (Figure 2). Moreover, Phloretin also caused in-

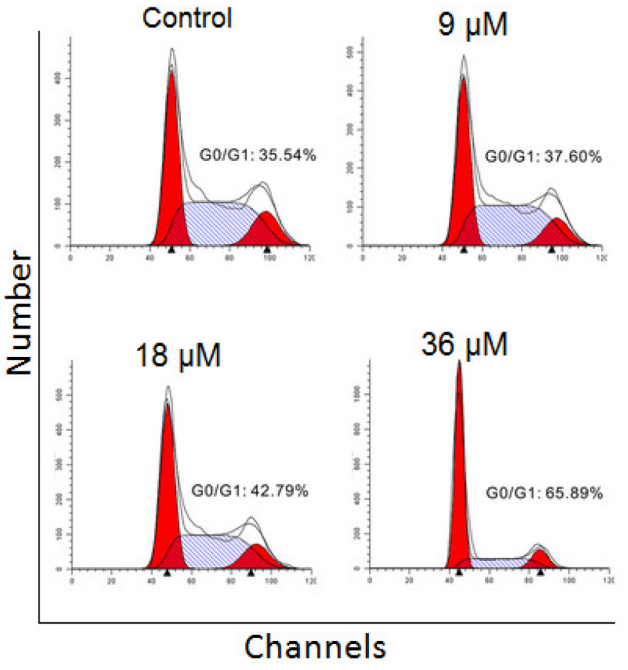

Figure 4. Flow cytometry showing Phloretin induces G0/G1 cell cycle arrest in SNU-1 gastric cancer cells. The experiments were performed in triplicate.

crease in the protein levels of Beclin-1, indicative of autophagy. Nonetheless, no apparent effects were observed on the LC3B-I expression (Figure 3).

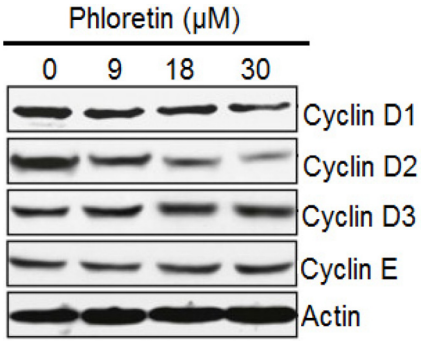

Figure 5. Effect of Phloretin on the cell cycle-related proteins as depicted by western blot analysis. The Figure shows that Phloretin suppresses cyclin D1 and cyclin D2 dose-dependently without any effect on cyclin D3 and cyclin E. The experiments were performed in triplicate.

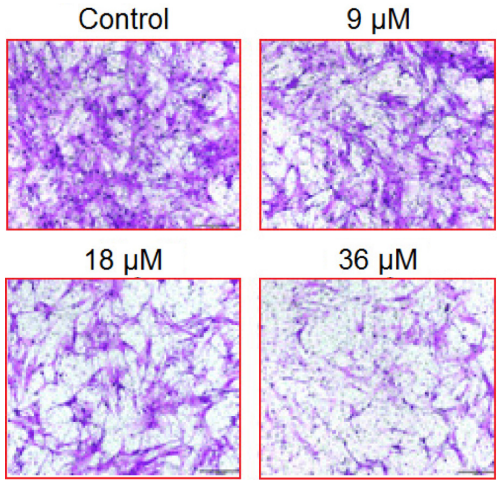

Figure 6. Effect of Phloretin on the invasion of the SNU-1 cells as depicted by transwell assay. The Figure shows that Phloretin inhibits the invasion of SNU-1 cells. The experiments were performed in triplicate.

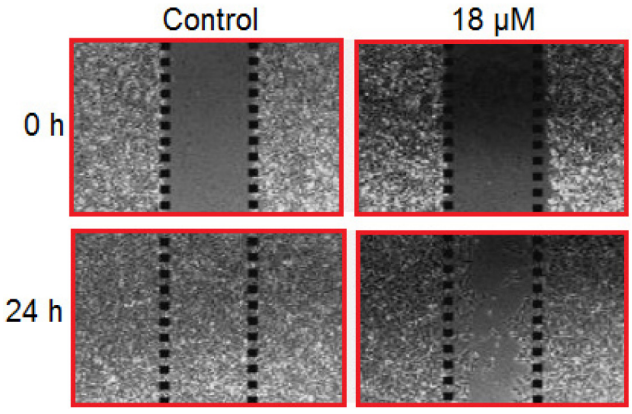

Figure 7. Wound healing assay showing that Phloretin inhibits the migration of SNU-1 cells. The experiments were performed in triplicate.

Phloretin causes G0/G1 arrest of gastric cancer cells

The SNU-1 gastric cancer cells were treated with various concentrations of Phloretin and the distribution of SNU-1cells at each phase of the cell cycle was determined by flow cytometry. The results showed that the G0/G1 phase cells increased remarkably upon Phloretin treatment. The percentage of G0/G1 phase cells were 35.54, 37.60, 42.79 and 65.89% at 0, 9, 18 and 36 μM concentrations of Phloretin respectively, suggestive of G0/G1 arrest of the SNU-1 cells (Figure 4). Western blot analysis was also performed to examine the effects of Phloretin on cell cycle-related proteins. The results showed that Phloretin inhibited the expression of cyclin D1 and D2. However, no significant effects were observed on the expression of cyclin D3 and E (Figure 5).

Phloretin inhibits the migration and invasion of the gastric cancer cells

The effects of Phloretin were also examined on the invasion and migration of the SNU-1 gastric cancer cells by transwell and wound healing assay. The results showed that Phloretin caused remarkable decrease in the invasion of the SNU-1 gastric cancer cells in a concentration-dependent manner (Figure 6). Moreover, wound healing assay showed that the migration of the SNU-1 cells was also decreased as evidenced from the wound width (Figure 7).

Phloretin blocks the ERK1/2/MAPK signalling pathway

The effects of Phloretin were also investigated on the ERK1/2 /MAPK signalling pathway at 0, 9, 18 and 36 μM. The results showed that Phloretin caused dose-dependent inhibition of the ERK1/2 and MAPK p38 phosphorylation. Nonetheless, the total ERK1/2 and MAPK p38 remained almost constant (Figure 8).

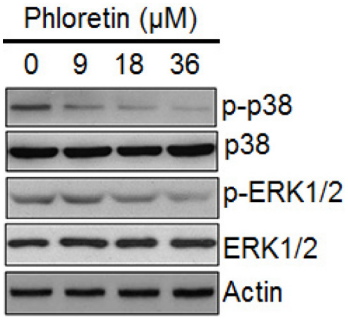

Figure 8. Western blot analysis showing the effect of Phloretin on the ERK1/2/MAPK signalling pathway in SNU-1 cells. The Figure shows that Phloretin blocks the phosphorylation of p38 and ERK 1/2 dose-dependently. The experiments were performed in triplicate.

Discussion

Although there have been recent improvements in the overall survival of gastric cancer patients, gastric cancer still causes enormous mortality worldwide [12]. For instance, half of the cancer cases detected in East Asian countries are gastric cancers [13]. Herein, we investigated the anticancer effects of Phloretin on human gastric cancer cells and the results showed that this molecule suppresses the proliferation rate of the human gastric cancer cells. However, the anticancer effects of Phloretin were significantly lower against the normal cells, suggesting that Phloretin selectively targets cancer cells. These results are well supported by previous studies wherein Phloretin has been shown to inhibit the growth of colon cancer cells [14]. Similarly, in another study, Phloretin was found to suppress the proliferation of breast cancer cells via induction of apoptosis [15]. Against this background, we sought to unveil the molecular mechanisms responsible for the anticancer effects of this molecule. Consistently, electron microscopic analysis of the Phloretin-treated SNU-1 cells was performed and the results showed that this molecule caused growth inhibition of the SNU-1 cells via induction of autophagy which was also accompanied with upregulation of LC3B II and Beclin-1 expression. These observations confirm previous investigations wherein molecules of plant-origin have been reported to cause autophagy in cancer cells, for example, Resveratrol has been reported to induce autophagy in colon cancer cells [16]. Moreover, Phloretin has been previously reported to trigger cell cycle arrest of glioblastoma cells [17], while our study found that Phloretin caused arrest of cancer cells in G0/G1 phase of the cell cycle, which was concomitant with decrease in Cyclin D1 and D2. This molecule has been reported to inhibit the metastasis of the human breast cancer cells [18]. Therefore, we also examined the anti-metastatic potential of Phloretin on the SNU-1 gastric cancer cells and found that this molecule inhibited the migration and invasion of gastric cancer cells, indicative of the potential of Phloretin as anticancer agent. ERK1/2 MAPK signal transduction has been shown to exhibit therapeutic implications [12] in the treatment of gastric cancer and herein we also found that Phloretin blocks this pathway in the human gastric cancer cells.

Conclusion

The results of the current study revealed that Phloretin inhibited the growth of human gastric cancer cells via induction of autophagy and cell cycle arrest. Phloretin also inhibited the migration and invasion of human gastric cancer cells, indicative of the potential of Phloretin as a lead molecule for gastric cancer treatment.

Acknowledgement

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Conflict of interests

The authors declare no conflict of interests.

References

1. Croce CM, Calin GA. miRNAs, cancer, and stem cell division. Cell 2005;122:6-7.
2. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell 2009;136:642-55.
3. Slaby O, Svoboda M, Fabian P et al. Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. Oncology 2007;72:397-402.
4. Shakya AK. Medicinal plants: future source of new drugs. Int J Herbal Med 2016;4:59-64.
5. Behzad S, Sureda A, Barreca D, Nabavi SF, Rastrelli L, Nabavi SM. Health effects of phloretin: from chemistry to medicine. Phytochem Rev 2017;16:527-33.
6. Ma L, Wang R, Nan Y et al. Phloretin exhibits an anticancer effect and enhances the anticancer ability of cisplatin on non-small cell lung cancer cell lines by regulating expression of apoptotic pathways and matrix metalloproteinases. Int J Oncol 2016;48:843-53.
7. Kobori M, Iwashita K, Shinmoto H, Tsushida T. Phloretin-induced apoptosis in B16 melanoma 4A5 cells and HL60 human leukemia cells. Biosci Biotechnol Biochem 1999;63:719-25.
8. Wu CH, Ho YS, Tsai CY et al. In vitro and in vivo study of phloretin-induced apoptosis in human liver cancer cells involving inhibition of type II glucose transporter. Int J Cancer 2009;124:2210-9.
9. Mojzis J, Varinska L, Mojzisova G et al. Antiangiogenic effects of flavonoids and chalcones. Pharmacol Res 2008;57:259-65.
10. Becker MS, Müller PM, Bajorat J et al. The anticancer phytochemical rocaglamide inhibits Rho GTPase activity and cancer cell migration. Oncotarget. 2016;7:51908.

11. Kinkade CW, Castillo-Martin M, Puzio-Kuter A et al. Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model. J Clin Invest 2008;118:3051-64.
12. Ang TL, Fock KM. Clinical epidemiology of gastric cancer. Singapore Med J 2014;55:621.
13. Correa P. Diet and gastric cancer. In: Diet, Nutrition and Cancer: A Critical Evaluation, CRC Press 2018; pp 1-10.
14. Park SY, Kim EJ, Shin HK et al. Induction of apoptosis in HT-29 colon cancer cells by phloretin. J Medicinal Food 2007;10:581-6.
15. Kim MS, Kwon JY, Kang NJ, Lee KW, Lee HJ. Phloretin induces apoptosis in H-Ras MCF10A human breast tumor cells through the activation of p53 via JNK and p38 mitogen-activated protein kinase signaling. Ann New York Academy Sci 2009;1171:479-83.
16. Miki H, Uehara N, Kimura A et al. Resveratrol induces apoptosis via ROS-triggered autophagy in human colon cancer cells. Int J Oncol 2012;40:1020-8.
17. Liu Y, Fan C, Pu L et al. Phloretin induces cell cycle arrest and apoptosis of human glioblastoma cells through the generation of reactive oxygen species. J Neuro-Oncol 2016;128:217-23.
18. Wu KH, Ho CT, Chen ZF et al. The apple polyphenol phloretin inhibits breast cancer cell migration and proliferation via inhibition of signals by type 2 glucose transporter. J Food Drug Analysis 2018;26:221-31.