ORIGINAL ARTICLE

Anticancer activity of Phloretin against the human oral cancer cells is due to G0/G1 cell cycle arrest and ROS mediated cell death

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Summary

Purpose: Phloretin is one of the important polyphenolics abundantly present across the plant kingdom. Studies have reported the anticancer effects of Phloretin against different human cancer cells. Nonetheless, the anticancer effects of Phloretin have not been explored against the human oral cancer cells. Therefore this study was designed to investigate the anticancer effects of Phloretin against the human oral cancer cells.

Methods: CCK-8 assay was used for the determination of cell viability. Annexin V/propidium iodide (PI) staining and flow cytometry were used for necrosis detection and cell cycle analysis, respectively. Wound healing assay was used for cell migration analysis. Western blot analysis was used for protein expression analysis.

Results: The results showed that Phloretin suppressed the proliferation rate of the human SCC-1 oral cancer cells and showed an IC_{50} of 12.5 μM. Nonetheless, Phloretin had negligible effects on the proliferation rate of the EBTr normal oral cells. DAPI staining showed that Phloretin did not induce

apoptosis and western blot showed that it had no apparent effects on the Bax and Bcl-2 expression. Nonetheless annexin V/PI staining showed that Phloretin caused cell death in SCC-1 oral cancer cells. Flow cytometric analysis showed that Phloretin caused increase in the reactive oxygen species (ROS) levels of the SCC-1 cells in a time and dose-dependent manner. Cell cycle analysis showed that Phloretin caused increase in the percentage of the SCC-1 cells in the G0/G1 phase of the cell cycle leading to G0/G1 cell cycle arrest. The G0/G1 arrest of SCC-1 cells was also associated with depletion of cyclin D1, CDK4 and CDK6 expression. Wound healing assay was also performed which showed that Phloretin suppressed the migration of the SCC-1 oral cancer cells, indicative of the anti-metastatic potential of Phloretin.

Conclusion: Phloretin exhibits significant growth inhibitory effects on the human oral cancer cells and may prove beneficial in oral cancer treatment.

Key words: oral cancer, phloretin, necrosis, cell migration, cell cycle arrest

Introduction

Considered as the 6th most common type of cancer, oral cancer causes significant mortality across the globe, especially in developing countries [1]. The 62% 5-year survival for oral cancer is still far from decent when compared to 5-year survival rates of other cancers such as breast (90%) and prostate cancer (99%) [2]. Therefore, in order to curb the incidence of oral cancer, it is important

to develop effective treatment strategies. Plants are sophisticated natural chemical factories with the potential to synthesize a wide diversity of chemical scaffolds [3]. This study was designed to investigate the anticancer effects of Phloretin against the human oral cancer cells and attempted to explore the underlying mechanisms. Phloretin is a plant secondary metabolite with enormous

pharmacological potential [4]. This molecule has been reported to cause remarkable suppression of lung cancer cells [5]. In another study, Phloretin has been reported to promote apoptosis in human leukemia cells [6]. Phloretin has been observed to suppress the expression of type II glucose transporter to halt the growth of liver cancer cells [7]. Moreover, the sensitivity of human lung cancer cells to cisplatin is increased by Phloretin [8]. On the other hand, studies have shown that Phloretin exhibits low or negligible toxic effects on normal cells [9]. Herein, we found that Phloretin exhibits significant anticancer effects on the human SCC-1 oral cancer cells with limited toxicity on the normal EBTr cells. The anticancer effects were found to be mainly due to cycle arrest of the cancer cells which was also accompanied by increased expression of cyclin D1. DAPI and annexin V/PI staining assays showed that Phloretin did not induced apoptosis in the SCC-1 oral cancer cells but showed that this molecule may inhibit the growth of oral cancer cells via ROS-mediated cell death. Cell migration is the first step that enables the cancer cells to move to other body parts and form metastasis [10]. Herein it was found that Phloretin suppressed the migration of oral cancer cells. The findings of the present study suggest that Phloretin may prove a useful lead molecule and semi-synthetic chemistry approaches may be used to develop more potent anticancer agents

Methods

Cell counting kit-8 (CCK-8) assay

The SCC-1 and HTRET-OME cells were seeded in 96-well plates at a density of 4×10^5/well. After incubation for 24 h, 10µl CCK-8 (Dojindo Laboratories, Kumamoto, Japan) were added to the culture plates, and the plates were incubated for 2h at 37°C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

DAPI staining assay

The SCC-1 cells (0.6×10^6) were cultured in 6-well plates and treated with Phloretin at concentrations of 0, 7.5, 15 and 30 µM for 24h at 37°C. Subsequently, 25 µl of cell culture were put onto glass slides and stained with DAPI. The slides were then cover-slipped and examined under fluorescence microscope.

Annexin V/PI staining assay

ApoScan kit was used to determine the apoptotic SCC-1 cell percentage. In brief, Phloretin-treated SCC-1 cells (5×10^5 cells per well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC or PI. The percentage of apoptotic SCC-1 cells at each concentration was then determined by flow cytometry.

Cell cycle analysis

The SCC-1 cells treated with 0.25% trypsin were centrifuged at 1000 r/min for 5 min to remove the Dulbecco's Modified Eagle's Medium (DMEM). The cells were washed with phosphate buffered saline (PBS), then
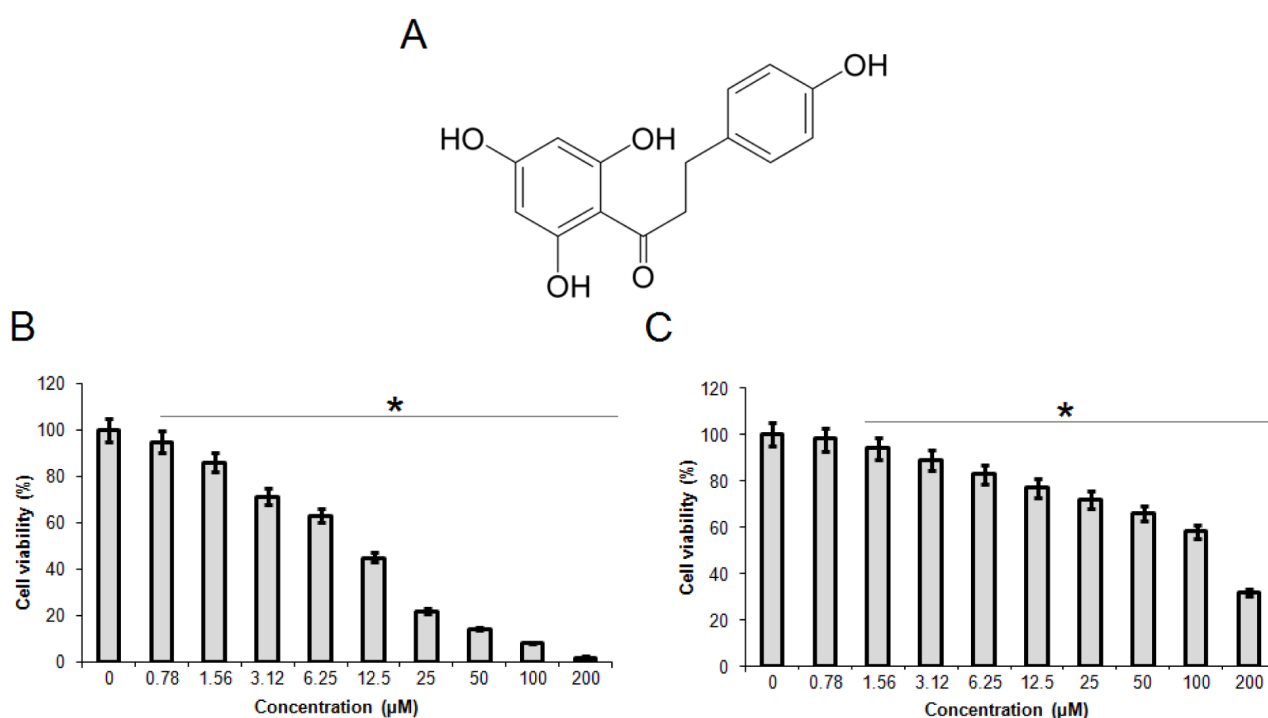

Figure 1. A: Chemical structure of Phloretin. Effect of Phloretin on the viability of (B) SCC-1 oral cancer cells and (C) hTRET-OME normal cells. The experiments were carried out in triplicate and shown as mean ± SD (*p<0.05).

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 PI at 37°C for 30 min in the dark. Cell cycle was detected by flow cytometry and expressed as the percentage of cells in each phase of the cell cycle.

Wound healing assay

In brief, SCC-1 cells were cultured in 6-well plates for 24 h. Then, after making a scratch line on the cells using a 200 µl sterile pipette tip, the plates were incubated at 37°C in 5% CO$_2$. Wound healing was observed at 0 and 24 h using an inverted microscope.

Western blot analysis

The SCC-1 cells were then lysed in lysis buffer containing protease inhibitor. Around 45 µg of proteins from each sample were subjected to separation on 10% SDS-PAGE, followed by transferring the proteins to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room tempera-

ture 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 protein signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

Statistics

The experiments were performed in triplicate and the values are the mean of the 3 repeat experiments ± SD. Significant differences were graded as *p<0.05, **p<0.01 and ***p<0.001. Student's t-test using GraphPad prism 7 software was employed for statistical analyses.

Results

Phloretin decreases the growth of oral cancer cells

The CCK8 assay was used to demonstrate the effects of Phloretin on the growth of the SCC-1 oral cancer and HTRET-OME normal cells. Phloretin caused a significant reduction in the proliferation rate of the SCC-1 cells. The effects of Phloretin on the proliferation rate of the SCC-1 cells were concentration-dependent and the IC$_{50}$ was 12.5µM (Figure 1A). Interestingly, the effects of Phloretin on the normal HTRET-OME cells were less and an IC$_{50}$ of>100 µM was reported for Phloretin against these normal cells (Figure 1A). Microscopic analysis was also carried out to see the effects of Phloretin on the morphology of SCC-1 cells, which showed that Phloretin caused remarkable changes
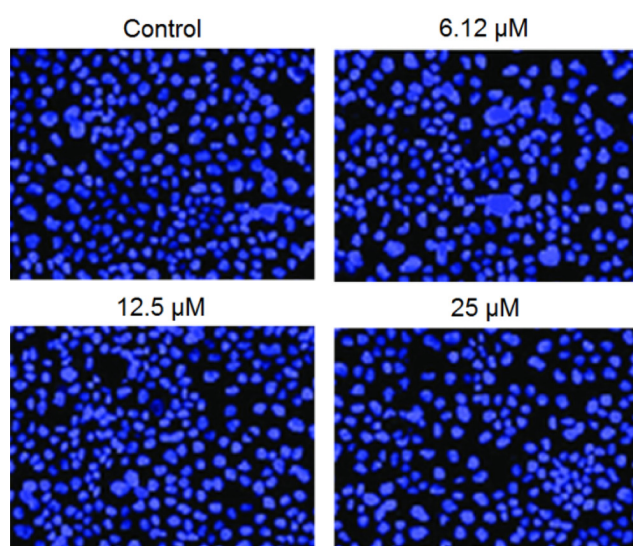

Figure 2. DAPI staining showing the effect of Phloretin on the nuclear morphology of SCC-1 oral cancer cells. The figure reveals that Phloretin did not induce apoptosis in SCC-1 cells. The experiments were performed in triplicate.
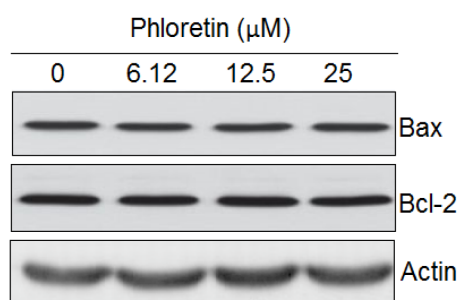

Figure 3. Western blot showing that Phloretin does not affect the expression of Bax and Bcl-2 at indicated concentrations. The experiments were performed in triplicate.
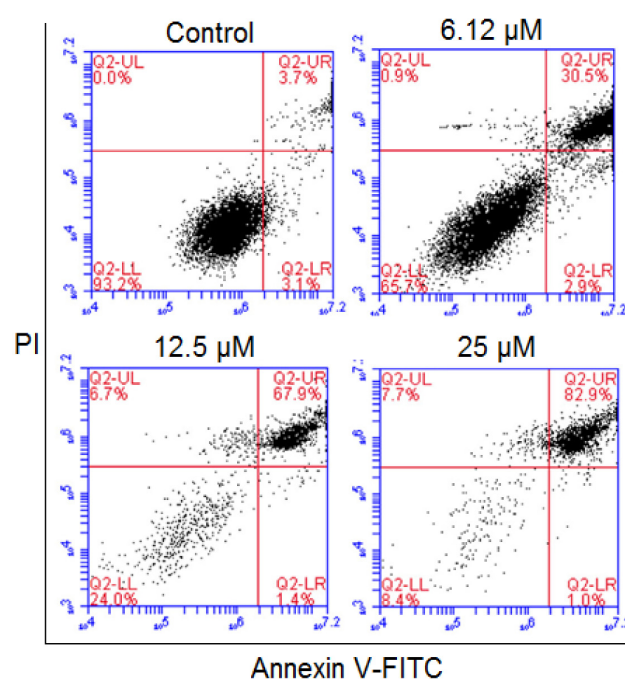

Figure 4. Annexin V/PI staining showing Phloretin-induced cell death in the SCC-1 oral cancer cells. The experiments were performed in triplicate.

in the morphology of the SCC-1 cells which included rounding, membrane blebbing and shrinkage (Figure 1B)

Phloretin triggers ROS-mediated cell death in oral cancer cells

To determine the underlying mechanism for the growth inhibitory effects of Phloretin, the SCC-1 cells were treated with different doses of this molecule and then stained with DAPI. The results of DAPI showed that Phloretin did not induce apo-

ptosis in SCC-1 cells (Figure 2). Next, western blot analysis was performed to investigate the effects of Phloretin on Bax and Bcl-2 expression. The results showed that this molecule did not cause any apparent changes on the expression of Bax and Bcl-2 (Figure 3). Nonetheless, the annexin V/PI staining showed that Phloretin caused necrosis in the SCC-1 oral cancer cells and the percentage of the necrotic cells increased with increase in the concentration of Phloretin. The percentages of the necrotic SCC-1 cells were 3.7, 30.5, 67.9, and 82.9% at 0, 6.12, 12.5 and 25 µM concentrations of Phloretin (Figure 4). Also, the results showed that Phloretin caused increase in the ROS levels in SCC-1 cells in a dose-dependent manner. The percentage of ROS was 100, 134, 168 and 199% at 3.7, 30.5, 67.9, and 82.9% at 0, 6.12, 12.5 and 25 µM concentrations of Phloretin (Figure 5A). The ROS production in SCC-1 cells was also increased time-dependently. The ROS levels were 100, 126, 158 and 195% at 0, 12, 24 and 48 h time intervals (Figure 5B).
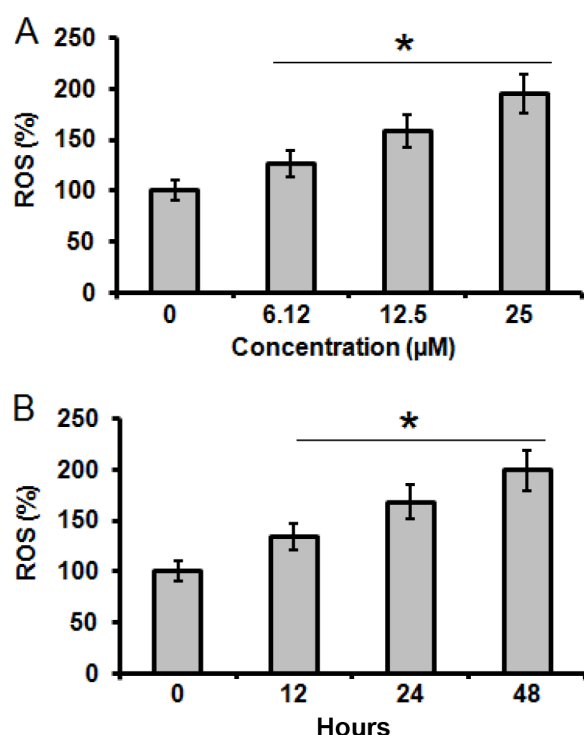

Figure 5. Effect of Phloretin on ROS at **A** different concentrations and **B** different time points. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Figure 7. Western blot analysis showing the effect of different concentrations of Phloretin on the expression of cell cycle related proteins. The Figure shows that the expression of CDK4, CDK6 and Cyclin D1 decreased upon Phloretin treatment. The experiments were performed in triplicate.
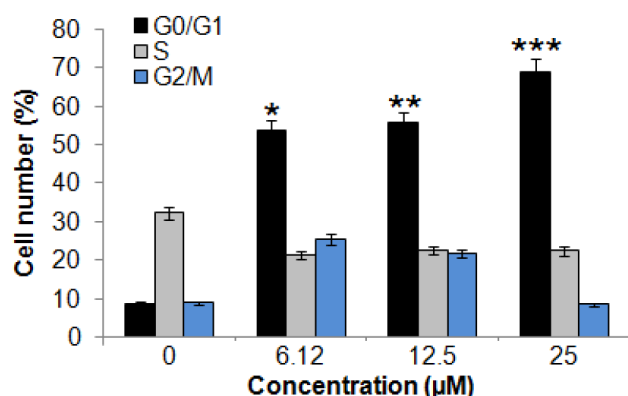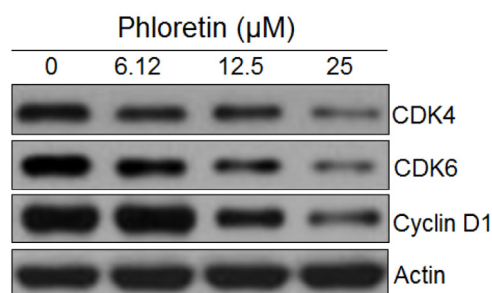

Figure 6. Effect of Phloretin on cell cycle phase distribution of the SCC-1 cells at indicated concentrations. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05), **p<0.01, ***p<0.001).
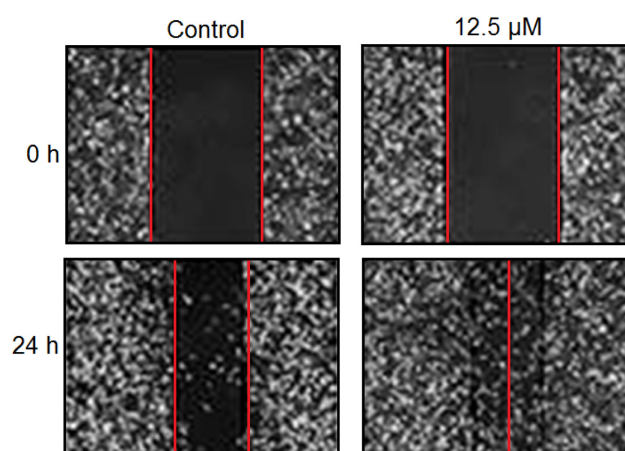

Figure 8. Effect of Phloretin at IC$_{50}$ on the migration of the SCC-1 oral cancer cells as depicted by wound healing assay. The results revealed that Phloretin inhibited considerably the migration of SCC-1 oral cancer cells. The experiments were performed in triplicate.

Phloretin causes G0/G1 arrest of oral cancer cells

The SCC-1 oral cancer cells were treated with various concentrations of Phloretin and their distribution 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 8.7, 53.7, 59.8 and 69% at 0, 6.12, 12.5 and 25 μM concentrations of Phloretin respectively, suggestive of G0/G1 arrest of the SCC-1 cells (Figure 6). Western blot analysis was also performed to examine the effects of Phloretin on cell cycle related proteins, which showed that Phloretin inhibited the expression of cyclin D1, CDk4 and CD6 concentration-dependently (Figure 7).

Phloretin inhibits the migration of the oral cancer cells

The effects of Phloretin were also examined on the migration of the SCC-1 oral cancer cells by wound healing assay. The results showed that this molecule caused remarkable decrease in the migration of the SCC-1 oral cancer cells as evidenced from the wound width (Figure 8).

Discussion

Natural products have provided humankind with diverse and novel chemical scaffolds that have been utilised for the treatment of human diseases such as cancer [11]. Oral cancer is one of the fatal malignancies responsible for remarkable morbidity and mortality [12]. Herein we investigated the anticancer effects of Phloretin on human oral cancer cells. The results showed that Phloretin suppresses the proliferation rate of human oral cancer cells. However, the anticancer effects of Phloretin were remarkably lower against the normal oral cells, suggesting that Phloretin selectively targets cancer cells. These observations are in agreement with previous investigations wherein Phloretin has been reported to suppress the growth of colon cancer cells [13]. In another study Phloretin was found to inhibit the growth of breast cancer cells via induction of apoptosis [14]. Keeping this in view, we sought to investigate whether Phloretin also inhibits the oral cancer growth via induction

of apoptosis. Consistently, DAPI staining was performed which showed that Phloretin did not induce apoptosis in the human oral cancer cells and also did not cause any alteration in the Bax/Bcl-2 ratio. Nonetheless, annexin V/PI staining showed that Phloretin caused necrosis of the SCC-1 oral cancer cells in a dose-dependent manner. These observations are in concordance with previous investigations wherein molecules of plant origin have been reported to cause cell death, for example, Caffeic acid n-butyl ester has been reported to induce necrosis in human lung cancer cells and hesperidin has been shown to induce necrosis in human skin cancer cells [15,16]. Moreover, ROS has been shown to play an important role in death of cancer cells [17]. Herein, we also examined the ROS levels in the Phloretin-treated oral cancer cells. The results showed that this molecule caused increase in the ROS levels in a concentration and dose-dependent manner. Phloretin has also been shown to cause cell cycle arrest of gastric cancer cells [18]. Hence, we also examined the effects of Phloretin on the cell cycle in human oral cancer cells. What it was found was that Phloretin caused arrest of cancer cells in G0/G1 phase of the cell cycle which was concomitant with increase in Cyclin D1, CDK4 and CDK6. This molecule has been reported to inhibit the metastasis of human breast cancer cells [19]. Therefore, we also examined the antimetastatic potential of Phloretin on the SCC-1 oral cancer cells and found that it inhibited the migration of the oral cancer cells, indicative of the potential of Phloretin as anticancer agent

Conclusion

The findings of the present study showed that Phloretin suppressed the proliferation rate of the human oral cancer cells via induction of cell cycle arrest and cell death. Phloretin also suppressed the migration of the human oral cancer cells suggestive of potential of Phloretin as the lead molecule for oral cancer treatment.

Conflict of interests

The authors declare no conflict of interests.

References

1.   Manikandan M, Rao AK, Arun kumar G et al. Oral squamous cell carcinoma: microRNA expression profiling and integrative analyses for elucidation of tumorigenesis mechanism. Mol Cancer 2016;15:28-32.

2.   Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018;68:7-30.

3.   Shakya AK. Medicinal plants: future source of new drugs. Int J Herbal Med 2016;4:59-64.

4. 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.

5. 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.

6. 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.

7. 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.

8. Ma L, Wang R, Nan Y, Li W, Wang Q, Jin F. 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.

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. Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell carcinoma—an update. CA Cancer J Clin 2015;65:401-21.

12. García-Martín JM, Varela-Centelles P, González M, Seoane-Romero JM, Seoane J, García-Pola MJ. Epidemiology of Oral Cancer. In: Oral Cancer Detection 2019, pp 81-93. Springer, Cham.

13. 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.

14. 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. Annals New York Academy of Sciences 2009;1171:479-83.

15. Zhang YX, Yu PF, Gao ZM, Yuan J, Zhang Z. Caffeic acid n-butyl ester-triggered necrosis-like cell death in lung cancer cell line A549 is prompted by ROS mediated alterations in mitochondrial membrane potential. Eur Rev Med Pharmacol Sci 2017;21:1665-71.

16. Zhao W, Chen Y, Zhang X. Hesperidin-triggered necrosis-like cell death in skin cancer cell line A431 might be prompted by ROS mediated alterations in mitochondrial membrane potential. Int J Clin Exp Med 2018;11:1948-54.

17. Chen D, Cao J, Tian L, Liu F, Sheng X. Induction of apoptosis by casticin in cervical cancer cells through reactive oxygen species-mediated mitochondrial signalling pathways. Oncol Rep 2011;26:1287-94.

18. Xu M, Gu W, Shen Z, Wang F. Anticancer Activity of Phloretin Against Human Gastric Cancer Cell Lines Involves Apoptosis, Cell Cycle Arrest, and Inhibition of Cell Invasion and JNK Signalling Pathway. Med Sci Monitor 2018;24:6551.

19. 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.