Original Paper

Estrogen Receptor Beta-Mediated Proliferative Inhibition and Apoptosis in Human Breast Cancer by Calycosin and Formononetin

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Key Words

Calycosin • Formononetin • Breast cancer • Estrogen receptor β • Insulin-like growth factor 1 receptor • miR-375

Abstract

Background: Calycosin and formononetin are two main components of isoflavones. In our previous studies, we have respectively reported their antitumor activities on breast cancer cell MCF-7. To further investigate the feasibility of isoflavones in clinically treating breast carcinoma, here we specifically focused on the comparison between calycosin and formononetin, along with the relevant mechanism. **Methods:** ER-positive (MCF-7, T-47D) and ER-negative breast cancer cells (MDA-231, MDA-435) were respectively treated with calycosin or formononetin. Cell proliferation and apoptosis were measured by MTT assay and flow cytometry. mRNA levels of ER beta (ER β) and miR-375 were quantified by real-time PCR. Expression of ER β and insulin-like growth factor 1 receptor (IGF-1R), and activation of poly (ADP-ribose) polymerase 1 (PARP-1) were determined by Western blotting. **Results:** Both calycosin and formononetin impaired proliferation and triggered apoptosis of ER-positive breast cancer cells (MCF-7, T-47D) in a time- and dose-dependent manner, especially in the treatment with calycosin. However, no such effect was observed in ER-negative breast cancer cells, indicating the correlation between isoflavones-induced inhibitory effect and ERs. Thus calycosin and most sensitive MCF-7 cells were used to study the relevant signaling pathway. After the treatment of calycosin, ER β expression was significantly increased in MCF-7 cells, followed by decrease of IGF-1R, activation of PARP-1 cleavage and downregulation of miR-375. **Conclusion:** Calycosin has an advantage on inhibiting breast cancer growth in comparison with formononetin, which is obtained by ER β -mediated regulation of IGF-1R signaling pathways and miR-375 expression.

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Introduction

In women, breast cancer represents the most common cancer diagnosis around the world, with average increases of about 2% [1]. It thus appears urgent to establish new strategies for early detection and effective treatment of breast cancer, so as to alleviate the cancer burden. Fortunately, evidence from studies has confirmed the carcinogenic effects of steroidal estrogens in the development of breast cancer, suggesting the feasibility of antiestrogen therapy in inhibiting tumor malignancy and growth [2, 3]. Phytoestrogens, a class of plant-derived phenolic compounds, are structurally similar to mammalian estrogens, which determines their abilities to bind to estrogen receptors (ER)- α and ER- β and then mimic estrogen effects [4]. Nevertheless, epidemiologic studies reveal that there are fewer breast cancer cases in Asian women than Western women, partially owing to the higher consumption of phytoestrogens from soybean products in Asian countries [5]. The link between phytoestrogens intake and breast cancer prevention show that, after binding to the ERs, phytoestrogens could also exhibit antiestrogenic activity other than estrogenic properties.

Phytoestrogens are subdivided into four main classes: isoflavones, stilbenes, lignans, and coumestans [6]. In our previous studies, we have demonstrated that calycosin, a main component of isoflavones, possess anti-carcinogenic activities in human breast cancer cells MCF-7 at relative high concentration ($\geq 25 \mu\text{M}$) [7]. Remarkably, similar anticancer effect was also observed in another typical isoflavones formononetin. We found that formononetin significantly induced apoptosis of MCF-7 cells at concentration higher than $30 \mu\text{M}$ [8]. These results raise the possibility that some phytoestrogens, such as isoflavones, have potential to be clinically used for the prevention of breast cancer carcinogenesis. Therefore, to provide more valuable information about isoflavones-mediated anti-carcinogenic action, here we compared calycosin and formononetin in human breast cancer cells, and investigated the underlying mechanism. In addition, our previous studies mainly focused on the growth and proliferation of ER-positive MCF-7 cells. Considering the diversity and heterogeneity in breast cancer subtypes, another human ER-positive breast cancer cell line T-47D was detected in the present study, with ER-negative breast cancer cells MDA-231 and MDA-435 served as control.

Insulin like growth factor-1 receptor (IGF-1R) is a transmembrane tyrosine kinase receptor, and consist two α -chains and two β -chains [9]. By binding to its ligands, IGF-1 and IGF-2, IGF-1R activates the downstream signaling pathway, thereby contributing to cell proliferation and protection from apoptosis [10]. Previously, we have shown that IGF-1R/PI3K/Akt signaling pathway is involved in formononetin-induced anti-proliferative effects on MCF-7 cells [11]. So we expect the same mechanism to be responsible for inhibition of cell proliferation and apoptosis with treatment of other isoflavones like calycosin.

In brief, the aim of this study was to investigate the effects of isoflavones, calycosin and formononetin, on human breast cancer cells *in vitro*, together with the possible mechanism. The results demonstrated that both calycosin and formononetin exerted inhibitory effect on ER-positive cells but not ER-negative cells, implying that binding and activation of ER by isoflavones may be involved in its inhibition of breast cancer. Furthermore, decreased IGF-1R expression, reduced miR-375 level and subsequent activation of Poly (ADP-ribose) polymerase 1 (PARP-1) cleavage may act as downstream targets.

Materials and Methods

Cell culture

Human breast cancer ER-positive cells (MCF-7, T-47D) and ER-negative cells (MDA-231, MDA-435) were all obtained from ATCC (Manassas, VA, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), and incubated at 37°C in a humidified atmosphere with 5% CO_2 .

MTT assay

Calycosin ($C_{16}H_{12}O_5$) was purchased from Phytomarker Ltd (Tianjin, China), and formononetin ($C_{16}H_{12}O_4$) was purchased from Sigma-Aldrich Ltd (St. Louis, USA). Their stock solution was both prepared by dissolving in dimethyl sulfoxide (DMSO) and stored at 4°C for further use. Cell proliferation after treatment of calycosin and formononetin was tested by MTT assay. Four cell lines were seeded into 96-well plates (5×10^3 cells per well) for 12 h, then respectively exposed to various concentrations of calycosin or formononetin (0, 25, 50, 100 μ M). After 24, 48 and 72 h, cells were incubated with 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) for 4h and then lysed in DMSO. The optical density (OD) values were measured at 490nm using a plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Flow cytometry assay

After treatment with calycosin or formononetin (0, 25, 50 and 100 μ M) for 48 h, four cell lines were washed by PBS and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). Apoptotic cells were detected by a FACS Aria flow cytometer (Becton Dickinson). The early apoptotic cells are Annexin V-FITC positive and PI negative, while late apoptotic cells Annexin V-FITC and PI double-positive.

Real-time PCR assay

ER-positive MCF-7 cells were treated with calycosin or formononetin (0, 25, 50 and 100 μ M) for 48 h. Total RNA from cells was extracted with TRIzol and 10 ng was used for reverse transcription using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Life Sciences, USA). Next, quantification of ER β and miR-375 was measured by qRT-PCR with specific primers for ER β , miR-375, GAPDH and miRNA U6 using SYBR Green qPCR Master Mix (Fermentas, Life Sciences, USA). Here GAPDH and miRNA U6 were respectively used as control to calculate the relative expression level of ER β and miR-375.

Western blot assay

Considering the high similarity between calycosin and formononetin, here more effective calycosin (rather than formononetin) was applied to further investigate the possible mechanism of isoflavones in treating breast cancer. ER-positive MCF-7 cells were incubated with calycosin at concentrations of 0, 25, 50 and 100 μ M for 48 h or 50 μ M for 0, 12, 24, 48, 72 h. Then the protein samples were separately prepared in a lysis buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), followed by blocking with Tris-buffered saline containing 5% non-fat milk. The proteins of interest were respectively labeled by primary antibodies against ER β (1:500; Santa Cruz, CA, USA; catalog number SC-53494), IGF-1R (1:500; Abzoom biolabs, Dallas, TX, USA; catalog number AM3009), PARP-1 (1:1000; Cell Signaling, Danvers, MA, USA; catalog number 9542) and β -actin (1:1000; Santa Cruz, CA, USA; catalog number sc-47778). Each band intensity was quantified by Image pro plus 5.02 software and normalized to the intensity of endogenous β -actin band.

Statistical analysis

Data were represented as mean $\pm$ SD. One-way ANOVA tests was performed using SPSS 12.0 to compare the different treatments. A p-value <0.05 was considered statistically significant.

Results

Inhibited proliferation of ER-positive cells by calycosin and formononetin

To better evaluate the anti-proliferation effect of isoflavones, ER-positive human breast cancer cells (MCF-7, T-47D) and ER-negative cells (MDA-231, MDA-435) were incubated with calycosin or formononetin (0, 25, 50, 100 μ M) for 24, 48 and 72 h. The results showed that both calycosin and formononetin induced time- and dosage-dependent inhibition of proliferation in ER-positive MCF-7 and T-47D cells ($p < 0.05$), indicated as decreasing OD values, as shown in Table 1. However, growth suppression was not observed in ER-negative MDA-231 and MDA-435 cells, which suggested that the two isoflavones suppressed tumor

Table 1. Anti-proliferative effect of calycosin (CA) and formononetin (FR) in human breast cancer cells detected by MTT assay (mean $\pm$ SD). OD values were obtained from three independent experiments performed in triplicate. Compare with control vehicle (0 μ M) ** $p < 0.05$

		0h		24h		48h		72h	
		FR	CA	FR	CA	FR	CA	FR	CA
MCF-7	Control(0 μ M)	0.341 $\pm$ 0.018	0.335 $\pm$ 0.021	0.555 $\pm$ 0.022	0.561 $\pm$ 0.024	0.792 $\pm$ 0.024	0.834 $\pm$ 0.024	0.934 $\pm$ 0.017	0.924 $\pm$ 0.021
	25 μ M	0.333 $\pm$ 0.017	0.331 $\pm$ 0.018	0.501 $\pm$ 0.019**	0.489 $\pm$ 0.021**	0.592 $\pm$ 0.029**	0.555 $\pm$ 0.031**	0.777 $\pm$ 0.025**	0.612 $\pm$ 0.023**
	50 μ M	0.338 $\pm$ 0.022	0.332 $\pm$ 0.012	0.423 $\pm$ 0.019**	0.402 $\pm$ 0.022**	0.501 $\pm$ 0.026**	0.461 $\pm$ 0.028**	0.552 $\pm$ 0.027**	0.473 $\pm$ 0.031**
	100 μ M	0.329 $\pm$ 0.021	0.328 $\pm$ 0.019	0.394 $\pm$ 0.021**	0.343 $\pm$ 0.019**	0.412 $\pm$ 0.023**	0.365 $\pm$ 0.023**	0.391 $\pm$ 0.019**	0.312 $\pm$ 0.026**
T-47D	Control(0 μ M)	0.335 $\pm$ 0.019	0.333 $\pm$ 0.013	0.561 $\pm$ 0.019	0.567 $\pm$ 0.014	0.758 $\pm$ 0.021	0.761 $\pm$ 0.024	0.969 $\pm$ 0.025	0.975 $\pm$ 0.028
	25 μ M	0.328 $\pm$ 0.016	0.329 $\pm$ 0.012	0.532 $\pm$ 0.020	0.502 $\pm$ 0.015**	0.669 $\pm$ 0.025**	0.641 $\pm$ 0.021**	0.821 $\pm$ 0.021**	0.724 $\pm$ 0.023**
	50 μ M	0.332 $\pm$ 0.014	0.323 $\pm$ 0.012	0.459 $\pm$ 0.021**	0.423 $\pm$ 0.018**	0.553 $\pm$ 0.028**	0.523 $\pm$ 0.031**	0.601 $\pm$ 0.028**	0.531 $\pm$ 0.022**
	100 μ M	0.325 $\pm$ 0.015	0.321 $\pm$ 0.015	0.402 $\pm$ 0.019**	0.376 $\pm$ 0.019**	0.441 $\pm$ 0.027**	0.405 $\pm$ 0.025**	0.400 $\pm$ 0.023**	0.383 $\pm$ 0.026**
MDA-231	Control(0 μ M)	0.331 $\pm$ 0.017	0.335 $\pm$ 0.022	0.551 $\pm$ 0.020	0.547 $\pm$ 0.022	0.641 $\pm$ 0.025	0.632 $\pm$ 0.028	0.883 $\pm$ 0.021	0.899 $\pm$ 0.029
	25 μ M	0.329 $\pm$ 0.015	0.338 $\pm$ 0.019	0.527 $\pm$ 0.021	0.513 $\pm$ 0.029	0.611 $\pm$ 0.018	0.603 $\pm$ 0.022	0.862 $\pm$ 0.027	0.854 $\pm$ 0.026
	50 μ M	0.327 $\pm$ 0.020	0.331 $\pm$ 0.021	0.501 $\pm$ 0.019**	0.482 $\pm$ 0.021**	0.568 $\pm$ 0.024**	0.531 $\pm$ 0.022**	0.769 $\pm$ 0.021**	0.721 $\pm$ 0.031**
	100 μ M	0.327 $\pm$ 0.019	0.333 $\pm$ 0.021	0.487 $\pm$ 0.023**	0.455 $\pm$ 0.016**	0.512 $\pm$ 0.028**	0.499 $\pm$ 0.031**	0.693 $\pm$ 0.024**	0.679 $\pm$ 0.033**
MDA-435	Control(0 μ M)	0.328 $\pm$ 0.015	0.333 $\pm$ 0.015	0.532 $\pm$ 0.018	0.551 $\pm$ 0.021	0.655 $\pm$ 0.028	0.651 $\pm$ 0.035	0.899 $\pm$ 0.028	0.888 $\pm$ 0.034
	25 μ M	0.325 $\pm$ 0.013	0.327 $\pm$ 0.021	0.522 $\pm$ 0.020	0.528 $\pm$ 0.016	0.609 $\pm$ 0.022	0.611 $\pm$ 0.031	0.852 $\pm$ 0.028	0.834 $\pm$ 0.033
	50 μ M	0.331 $\pm$ 0.018	0.338 $\pm$ 0.022	0.499 $\pm$ 0.019	0.492 $\pm$ 0.022**	0.555 $\pm$ 0.027**	0.549 $\pm$ 0.028**	0.752 $\pm$ 0.019**	0.733 $\pm$ 0.029**
	100 μ M	0.326 $\pm$ 0.017	0.329 $\pm$ 0.019	0.469 $\pm$ 0.250**	0.474 $\pm$ 0.021**	0.511 $\pm$ 0.031**	0.509 $\pm$ 0.035**	0.701 $\pm$ 0.021**	0.689 $\pm$ 0.026**

Table 2. The percentage of apoptosis in human breast cancer cells after treatment with calycosin (CA) and formononetin (FR) determined by flow cytometry (mean $\pm$ SD). Apoptosis rates were obtained from three independent experiments performed in triplicate. Compare with control vehicle (0 μ M) ** $p < 0.05$

Group	MCF-7		T-47D		MDA-231		MDA-435	
	FR	CA	FR	CA	FR	CA	FR	CA
Control(0 μ M)	2.19 $\pm$ 0.52	2.35 $\pm$ 0.67	2.32 $\pm$ 0.44	2.11 $\pm$ 0.52	2.19 $\pm$ 0.39	2.04 $\pm$ 0.53	2.28 $\pm$ 0.45	2.56 $\pm$ 0.67
25 μ M	4.99 $\pm$ 0.89	5.34 $\pm$ 1.23	3.45 $\pm$ 0.56	4.68 $\pm$ 0.78	3.12 $\pm$ 0.63	3.21 $\pm$ 1.23	3.43 $\pm$ 0.52	3.42 $\pm$ 0.45
50 μ M	10.17 $\pm$ 0.92**	15.88 $\pm$ 1.45**	8.11 $\pm$ 0.22**	11.92 $\pm$ 1.34**	3.89 $\pm$ 0.76	3.78 $\pm$ 1.09	4.16 $\pm$ 0.67	4.63 $\pm$ 0.91
100 μ M	21.33 $\pm$ 0.77**	28.67 $\pm$ 0.99**	15.21 $\pm$ 0.82**	20.29 $\pm$ 1.21**	4.03 $\pm$ 0.56	4.11 $\pm$ 0.89	5.21 $\pm$ 0.32	4.59 $\pm$ 1.01

cell growth through regulation of ER-mediated signaling pathway. Moreover, we found that calycosin caused stronger inhibition of cell proliferation compared to formononetin, especially in MCF-7 cells.

Increased apoptosis of ER-positive cells by calycosin and formononetin

Consistent with results from MTT assay, both calycosin and formononetin promoted apoptosis of MCF-7 and T-47D cells in a dosage-dependent manner, leading to obvious increase in the percentage of early apoptotic cells ($p < 0.05$) (Table 2). Similarly, calycosin displayed greater proapoptotic function than formononetin in the same ER-positive cell lines. And after treatment of calycosin, MCF-7 cells always exhibited higher apoptotic rate than T-47D cells. On the contrary, there was no significant change in MDA-231 and MDA-435 cells treated with calycosin or formononetin. The increased apoptosis of ER-positive cells further confirmed the involvement of ER activation in isoflavones-mediated growth regulation of breast cancer cells.

Regulation of ER β and miR-375 in MCF-7 cells by calycosin and formononetin

Based on results from MTT assay and flow cytometry assay, we chose the most drug-sensitive MCF-7 cells to study the possible mechanism for isoflavones-induced growth

Table 3. Expression of ER β and miR-375 after treatment with calycosin (CA) and formononetin (FR) determined by real-time PCR (mean $\pm$ SD). Data were obtained from three independent experiments performed in triplicate. Compare with control vehicle (0 μ M) ** $p < 0.05$

Group	ER β / GAPDH		miR-375/U6	
	FR	CA	FR	CA
Control(0 μ M)	0.521 $\pm$ 0.032	0.521 $\pm$ 0.032	1.034 $\pm$ 0.032	1.034 $\pm$ 0.032
25 μ M	0.563 $\pm$ 0.043	0.753 $\pm$ 0.082**	1.023 $\pm$ 0.037	0.988 $\pm$ 0.032
50 μ M	9.012 $\pm$ 0.056**	1.032 $\pm$ 0.061**	0.792 $\pm$ 0.031**	0.721 $\pm$ 0.041**
100 μ M	1.004 $\pm$ 0.072**	1.267 $\pm$ 0.055**	0.612 $\pm$ 0.024**	0.511 $\pm$ 0.052**

Fig. 1. Upregulated ER β (A) and inhibited IGF-1R (B) expression in MCF-7 cells by calycosin. Cells were treated with various concentrations of calycosin (0, 25, 50, 100 μ M) for 48h, and then protein samples were labeled with specific antibodies against ER β and IGF-1R. The same blots were probed with anti- β -actin antibody as a loading standard. Results are representative of three independent experiments performed in triplicate. ** $p < 0.05$ versus control vehicle (0 μ M).

Fig. 2. Activated PARP-1 cleavage in MCF-7 cells by calycosin. Cells were treated with 0, 25, 50, 100 μ M calycosin for 48h (A), or 50 μ M for 0, 12, 24, 48, 72 h (B). The cleaved PARP-1 in MCF-7 cells was determined by Western blot, with β -actin served as loading controls. Results are representative of three independent experiments performed in triplicate. ** $p < 0.05$ versus control vehicle (0 μ M or 0h).

inhibition and apoptosis of breast cancer cells. The results showed that calycosin and formononetin gradually upregulated expression levels of ER β but downregulated expression levels of miR-375, which was again more obvious in calycosin-treated MCF-7 cells ($p < 0.05$), as shown in Table 3.

Activation of ER β and inactivation of IGF-1R in MCF-7 cells by calycosin

After detection of ER β mRNA levels in MCF-7 cells, the levels of ER β protein with treatment of calycosin (0, 25, 50 and 100 μ M) were determined by Western blot assay. We

found that, in accordance with mRNA data, calycosin significantly increased ER β expression in a dosage-dependent manner when compared with 0 μ M control vehicle ($p < 0.05$), as shown in Figure 1A. Meanwhile, the highest levels of IGF-1R were showed in untreated MCF-7 cells, whereas calycosin significantly reduced its expression ($p < 0.05$), especially at the highest concentration (100 μ M) (Fig. 1B).

Induction of PARP-1 cleavage in MCF-7 cells by calycosin

During cell apoptosis process, cleavage of PARP-1 by caspases is considered as an important hallmark. When MCF-7 cells were treated with 0, 25, 50 and 100 μ M calycosin for 48h or 50 μ M calycosin for 0h, 12h, 24h, 48h and 72h, it was found that levels of cleaved PARP-1 time- and dosage-dependently increased ($p < 0.05$), which suggests that activation of PARP-1 cleavage is involved in calycosin-mediated cell apoptosis, as shown in Figure 2A and Figure 2B. The reduced PARP-1 cleavage at 72h may be caused by gradual decline in concentration of calycosin.

Discussion

Calycosin and formononetin are two main components of isoflavones that have been attracting attention for their possible anti-cancer effects. Previously, we have separately reported that both of them significantly inhibited proliferation and induced apoptosis in human breast cancer MCF-7 cells at relative high concentration. Based on these findings, we here further observed and compared the anti-proliferation effect between calycosin and formononetin, so as to provide more valuable information in evaluating the feasibility of their clinical application. The results demonstrated that although the two isoflavones successfully decreased cell proliferation and triggered apoptosis in ER-positive cells MCF-7 and T-47D, the inhibitory effects on the same cell line were found greater in treatment of calycosin versus formononetin, implying that calycosin may be superior to formononetin in treating human breast cancer.

However, the specific mechanism associated with isoflavones-mediated anti-proliferation remains unclear. It is reported that the increase in circulating estrogen accounts for development of breast cancer, and the interaction between estrogen and ERs partially contributes to estrogen-mediated tumor growth [12]. Accordingly, regulation of ERs may be a promising target for treating estrogen-dependent breast cancer. In present study, not only ER-positive but ER-negative breast cancer cell lines were observed after treatment of calycosin and formononetin. We found that the two isoflavones both exerted anti-proliferation effects against ER-positive MCF-7 and T-47D, while there was no change in cell proliferation and apoptosis for ER-negative MDA-231 and MDA-435. Therefore, we concluded that calycosin and formononetin may exhibit anticancer properties through ER-dependent mechanism.

Until now, more and more studies have proved the function of ER α in malignancies, as well as the antagonistic relationship between ER α and ER β in modulating the development of breast cancer [13, 14]. We then determined ER β expression respectively at mRNA and protein levels. As expected, treatment with calycosin and formononetin significantly enhanced ER β mRNA levels in a dosage-dependent manner. In addition, there was corresponding increase in the synthesis of ER β protein after treatment of calycosin, again confirming that upregulation of ER β plays a major role in isoflavones-mediated inhibition of ER-positive breast cancer.

IGF-1R signaling could control cell growth and participate in the development of normal tissues and malignancy [15, 16]. At present, epidemiological studies have correlated increased IGF-1 serum levels with risk of breast cancer, so inhibition of IGF-1R signaling can serve as new target for breast cancer therapy [17, 18]. Especially, Tang et al. reported that estrogen activated the IGF-1R signaling through ER β in lung cancer and finally promoted A549 cell proliferation [19]. In agreement with their observation, we here demonstrated that, subsequent to calycosin-induced upregulation of ER β , the expression of IGF-1R gradually

declined. It means that the correlation of ER β and IGF-1R signaling pathway also exists in ER-positive breast cancer, which could be regulated by isoflavones.

Activation of caspase cascades, including cleavage of some key proteins, is considered responsible for cell apoptosis [20]. One of those substrates is PARP-1 that could be cleaved by caspase-3 into 89-kD and 24-kD fragments. And 24-kD PARP-1 fragment can block DNA repair, finally leading to cell death [21]. Hence cleaved PARP-1 is considered as apoptotic markers. We investigated whether activation of PARP-1 cleavage occurred during isoflavones-induced cell apoptosis. The results showed that next to ER β -mediated IGF-1R decline, there was increased cleavage of PARP-1 with the treatment of calycosin in a time- and dose-dependent manner, indicating the participation of PARP-1.

On the other hand, compelling studies have demonstrated that Micro RNAs (miRNAs), a class of small non-coding RNA, have a major impact on cell growth and differentiation by regulating gene expression at posttranscriptional level [22, 23]. Simonini et al. reported that miR-375 was expressed highly in breast cancer cells MCF-7, and there existed a positive loop between ER α and miR-375 [24]. Notably, our study revealed that along with reduced ER β levels, the miR-375 expression dose-dependently decreased with treatment of calycosin. Based on these data, we hypothesize that a negative loop between ER β and miR-375 may also be available in breast cancer cells, which could be strengthened by isoflavones. Nonetheless, further research was needed to confirm this hypothesis.

In a word, our study proved the anti-tumor activity of calycosin and formononetin, the two main isoflavones, in ER-positive breast cancer cells, even though the effect was somewhat weak for formononetin. Meanwhile, this anti-proliferative effect was achieved by upregulated ER β -mediated IGF-1R inhibition, PARP-1 cleavage activation and miR-375 decrease. Depending on these results, it can be predicted that isoflavones is a promising alternative for clinical therapy of breast cancer.

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