

Original Research

## The individual or combinational effects of Hesperetin and Letrozole on the activity and expression of aromatase in MCF-7 cells

S. T. Rahideh<sup>1</sup>, F. Shidfar<sup>1</sup>, M. Nourbakhsh<sup>2</sup>, M. Hoseini<sup>3</sup>, F. Koohdani<sup>4</sup>, M. Entezam<sup>5</sup>, M. Keramatipour<sup>5\*</sup>

<sup>1</sup>Department of Nutrition, School of Public Health, Iran University of Medical Sciences, Tehran, Iran

<sup>2</sup>Department of Biochemistry, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

<sup>3</sup>Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

<sup>4</sup>Department of Molecular and Cellular Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran

<sup>5</sup>Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

**Abstract:** Aromatase catalyzes the last and rate-limiting step in estrogen biosynthesis. Inhibition of estrogen production is a common strategy for breast cancer treatment. Citrus flavonoids have been confirmed to exhibit efficacious biological activities, particularly in cancer therapy. This study was carried out to investigate the effect of hesperetin on the activity and expression of aromatase and compare this property with letrozole as an aromatase inhibitor in MCF-7 breast cancer cell line. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assays in this study demonstrated that hesperetin at a concentration of 200  $\mu$ M decreased cell viability in a time dependent manner ( $P < 0.05$ ). Aromatase activity assay, based on  $17\beta$ -Estradiol (E2) production from testosterone, revealed that hesperetin had no effect. Real-time PCR results indicated that treatment with 1  $\mu$ M concentration of hesperetin for 48 h significantly decreased relative aromatase expression ( $P = 0.004$ ). Combination of letrozole and hesperetin also had no effect on aromatase. The changes in activity paralleled the expression of aromatase. Likely, the reduction in aromatase activity was delayed in time along with the reduction in expression ratio; however additional studies are needed to confirm this. In conclusion, the present study showed that hesperetin could decrease expression of aromatase at low concentrations in MCF-7 breast cancer cells.

**Key words:** Hesperetin, letrozole, enzyme activity, gene expression, aromatase, MCF-7 cells.

### Introduction

Breast cancer is the most frequent cancer among women and ranks second most common cancer (1.4 million cases, 10.9%) overall (1). Incidence rates of breast cancer vary from 19.3 per 100,000 women to 89.9 per 100,000 women both in developed and developing regions and are 28.1 per 100,000 women in Iran (1, 2). Although the precise mechanisms that promote breast cancer are not completely known, most breast cancer cases occur in postmenopausal women, and the majority of them are estrogen dependent (3). Estrogens cause breast cancer by stimulating cell growth and proliferation, and have been known as human carcinogens (4).  $17\beta$ -Estradiol (E2) is considered as a major risk factor for both initiation and promotion of breast cancer (5). This causal effect of estrogen exposure has been indicated in a transgenic animal model (6).

Aromatase is a member of the cytochrome P450 (CYP 19) enzyme family and catalyzes the aromatization reaction, the last and rate-limiting step in estrogen biosynthesis (4, 5, 7). Aromatase is encoded by a single gene *CYP 19A1* and plays a crucial role in the conversion of androstenedione to estrone (E1) and testosterone (T) to E2 (3, 4). Increased aromatase activity and expression have been demonstrated in breast cancer tissues (5, 7, 8). Hence nowadays, CYP19 inhibition is a common strategy for hormone-dependent breast cancer treatment (4, 7, 8). Aromatase inhibitors (AIs) have been developed as therapeutic agents for controlling estrogen receptor-positive breast cancer (9). Letrozole is one of the non-steroidal aromatase inhibitors (NSAIs) (10) and

suppress total-body aromatization more than 99% (11). The third-generation aromatase inhibitor like letrozole is widely used as the first-line therapy in the treatment of hormone-dependent breast cancer in postmenopausal women (10). The second class of NSAIs are natural products with the same inhibition activity on aromatase (7). Because many natural products such as the dietary polyphenols have low toxicity, they are potentially useful in chemopreventive therapy (4). Citrus flavonoids have shown a wide range of chemopreventive property, including antioxidant, anticarcinogenesis, antiangiogenesis, and apoptotic properties (12). Reports based on the *in vitro* action of citrus flavonoids have demonstrated anti-proliferative actions against cancer cells (13-15). Hesperetin, aglycone of hesperidin, is a member of the flavanone subclass of flavonoids, which is found in citrus fruits (16). Hesperetin treatment induces cell cycle arrest in G1-phase in MCF-7 cell line and has chemopreventive effect against 1,2-dimethyl hydrazine-induced rat colon carcinogenesis (16, 17). Hesperetin has also shown cytotoxic activity and induced apoptosis via activating caspase 3 HL-60 cells (12). Various types of flavonoids have been previously investigated for aromatase inhibition using microsomal assay (18),

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\* **Corresponding author:** Mohammad Keramatipour, Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Email: keramatipour@sina.tums.ac.ir

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but information about possible ability of hesperetin to act as AI is limited and controversial. Hesperetin inhibited microsomal aromatase activity (18), but increased CYP19 mRNA expression in MCF-7 breast cancer cell line (8). On the other hand, hesperetin inhibited aromatase activity in animal study (19). Therefore, the aim of current study was not only to evaluate the effect of hesperetin on aromatase activity and expression, but also

compare this function with letrozole.

## Materials and Methods

### Chemicals

The following reagents were obtained: hesperetin from Sigma-Aldrich (Germany); testosterone (purity  $\geq 98\%$ ) and dextran coated charcoal from Sigma Chemical Co. (St Louis, MO, USA); letrozole (purity  $\geq 99\%$ ) from Ind-Swift-India (India); RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA from Gibco BRL (Grand Island, NY, USA); phenol-red free RPMI medium and dimethyl sulfoxide (DMSO) from Biosera (UK) and Applichem (USA), respectively.

### Cell culture condition

Human ER- positive breast cancer cell line MCF-7 was received from the Avicenna Research Institute (Tehran, Iran). The cells were maintained in RPMI-1640, supplemented with 10% FBS, 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The cells were harvested with trypsin-EDTA when reaching 80% confluency. Subsequently, MCF-7 cells were plated onto 96-well plates (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT)) or 6-well plates (aromatase activity and mRNA expression). For treatment, the stock solutions of hesperetin and letrozole were prepared with DMSO and stored at -70 °C. Suitable amounts of stock solution of hesperetin and letrozole were added freshly into the medium to achieve the different concentrations (final DMSO concentration was < 0.1%). The experiment was repeated three times and each sample was applied in duplicate.

### Cell viability assay

In order to examine cytotoxic effects of hesperetin and letrozole, cell viability was determined by MTT assay, based on the capacity of MCF-7 cells to reduce MTT to formazan. Briefly, cells (5000 cells/well) were seeded in 96-well plates. After 24 h, the medium was replaced with phenol-red free RPMI medium supplemented with 2% dextran-coated charcoal-stripped FBS (DCC-FBS) and incubated overnight. Then the cells were incubated for 24, 48, and 72 h in the presence or absence of various concentrations of letrozole and hesperetin. After treatment, media were replaced by 100  $\mu$ L fresh media containing MTT solution (5 mg/mL) and the cells were incubated for 3 h at 37°C. Subsequently, MTT-containing media was removed and 100  $\mu$ L DMSO was added to each well. After gentle mixing, the absorbance was monitored in the wavelength range of 570 - 630 nm using a plate reader (BioTek, USA). The percentage of cell viability was estimated by comparing with the untreated control cells.

### Aromatase activity assay

Aromatase enzyme activity was determined by measuring the conversion of androgenic substrate testosterone to E<sub>2</sub> in cell culture (20). Briefly, MCF-7 cells (3  $\times 10^5$  cells/well) were plated in medium supplemented with 5% FBS and allowed to attach overnight. Then culture medium was aspirated and replaced by 2% DCC FBS phenol red-free RPMI medium containing 100 nM testosterone and with either vehicle (0.1% DMSO), or various concentrations of hesperetin, letrozole, or combination of letrozole and hesperetin for 48 h. E<sub>2</sub> concentrations in media were determined using an electrochemiluminescence immunoassay (ECLIA) kit (Roche, Germany) by Elecsys 2010 instrument. Protein content was assessed by the Bradford method, with bovine serum albumin as standard (21).

### Quantitative real-time PCR assay of aromatase expression

MCF-7 cells were treated for 48 h as described earlier. Following incubation, the cells were washed with PBS and total RNA was isolated using Ribospin kit (GeneAll, South Korea) according to manufacturer's instructions and stored at -70°C until analysis. The concentration and purity of the isolated RNA were determined at an absorbance wavelength of 260 and 280 nm using Nano Drop Spectrophotometer (ND-2000c) (Wilmington, Delaware USA) and by gel electrophoresis. Complementary DNA (cDNA) was synthesized from 1  $\mu$ g of total RNA with oligo dT and randomhexamer primers using HyperScript™ RT master mix (GeneAll, South Korea). Real-time PCR was performed by a Corbett rotor gene system (Qiagen, Germany), utilizing SYBR Green reagents (Ampliqon, Denmark). Primers sequences are as follows: CYP19 (forward: 5'-GAATTGGACCCCT-CATCTCCC-3', reverse: 5'-GCCGAATCGAGAGC-TGTAAT-3') and Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5'-GGGTGTGAA-CCATGAGAAGT-3', reverse: 5'-GGCATGGACTG-TGGTCATGA-3'). GAPDH was used as a normalizer. PCR reaction contained 200 nmol/L of forward and reverse primer, 1  $\mu$ l cDNA sample and the final reaction volume was 20  $\mu$ l. The reaction mixture was heated initially at 95 °C for 15 min, followed by 45 amplification cycles with 15 second (sec) denaturation at 95°C, 20 sec annealing at 62°C and 30 sec at 72°C for extension. PCR was performed in triplicate reactions. In each run, a melt curve was acquired to ensure the specificity of amplification. The threshold cycle (Ct) values were entered into the Relative Expression Software Tool (REST) and were analyzed for significant differences (P<0.05) by pair wise fixed reallocation randomization test using the following formula (1) for calculating expression ratio:

$$\text{Expression ratio} = \frac{(E_{\text{target}})^{\Delta C_{\text{t target}}}}{(E_{\text{reference}})^{\Delta C_{\text{t reference}}}} \quad (1)$$

Where E is the efficiency of reaction,  $\Delta C_{\text{t}}$  the crossing point difference of an unknown sample (treated) versus a control (untreated).

### Statistical analysis

Data were presented as means  $\pm$  standard error (SE) of duplicate cultures performed in at least three independent experiments. Statistical significance of diffe-

rences between samples were calculated by one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test or Student's t-test using the SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). P-value of less than 0.05 was considered statistically significant.

## Results

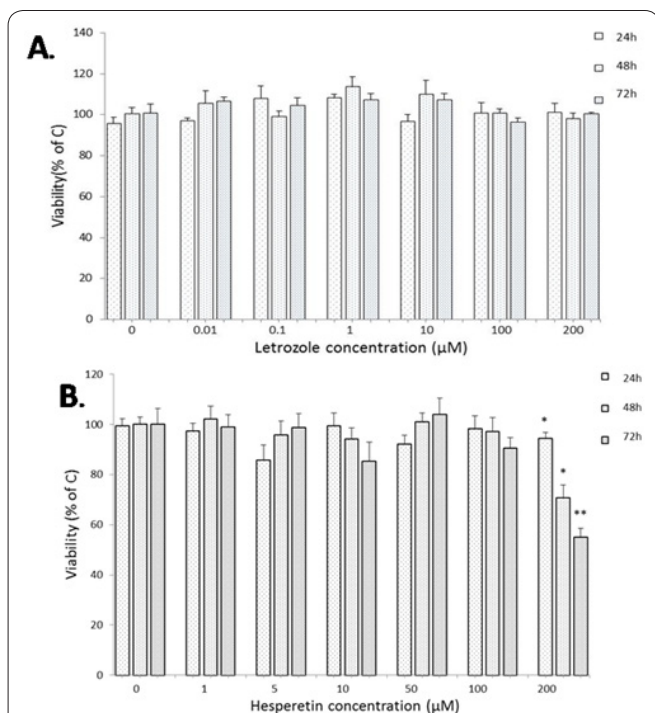
### Cell viability- MTT assay

The effect of hesperetin and letrozole on cell viability was measured using the MTT assay for 24, 48, and 72 h. As shown in figure 1A, letrozole did not have any significant effect on cell viability in comparison with control. Hesperetin at a concentration of 200  $\mu\text{M}$  decreased viability in a time dependent manner ( $P < 0.05$ ) and no effect of hesperetin was seen at other concentrations compared with control (figure 1B). Cell viability was also significantly decreased at 200  $\mu\text{M}$  concentration compared with other concentrations for hesperetin after 24, 48 and 72 h ( $P < 0.05$ ).

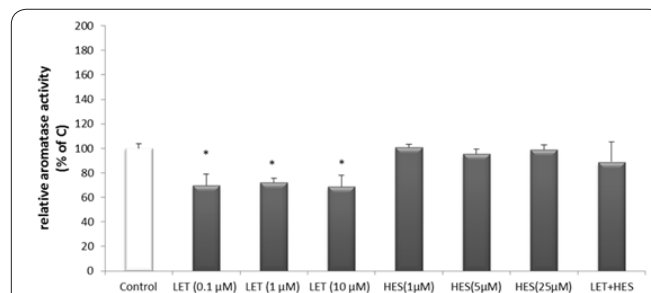
### Aromatase enzyme activity- estradiol assay

The effects of letrozole and hesperetin on aromatase activity are shown in figure 2. Aromatase enzyme activity was determined based on converting of testosterone to E2 over 48 h treatment with letrozole and hesperetin.

Letrozole significantly decreased aromatase activity at 0.1, 1 and 10 concentrations ( $P < 0.05$ ; figure 2). There was also a trend towards lower aromatase activity after treatment with hesperetin, nevertheless this decrease was not significant. Similarly, the combination of letrozole and hesperetin did not significantly alter aromatase activity (figure 2).



**Figure 1.** Effect of letrozole and hesperetin on MCF-7 cell viability compared with control. Cultured cells were treated without (control) and with (A) letrozole and (B) hesperetin under various concentrations for 24, 48 and, 72 h. Viability was determined by MTT assay. Values represent mean  $\pm$  SE. \*, \*\*significantly different from control at  $P \leq 0.001$  and  $P = 0.017$ , respectively.



**Figure 2.** Aromatase activity as the percentage of control ( $\pm$ SE) after incubation of MCF-7 cells for 48 h with 100 nM testosterone without (control) or with various concentrations of letrozole (LET), hesperetin (HES) and both (LET+HES). \* Significantly different from control at  $P < 0.05$ .

### Aromatase expression ratio- real-time PCR assay

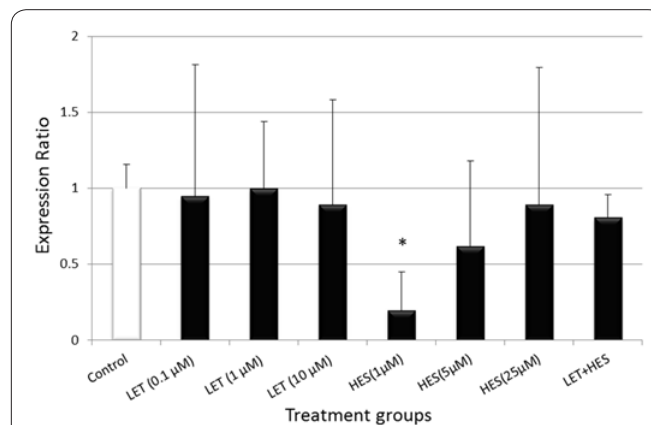
Expression of aromatase gene was determined using quantitative real-time PCR.

As seen in figure 3, 48 h treatment with low dose (1  $\mu\text{M}$ ) of hesperetin significantly ( $P = 0.004$ ) down-regulated aromatase gene expression compared to untreated cells. No changes in the aromatase expression were observed upon exposure to either letrozole or combination of letrozole and hesperetin compared with control (figure 3).

## Discussion

Bioactive food components such as phytochemicals are being investigated for the prevention of both estrogen receptor-positive and negative breast cancers (10). The findings of this study indicated that hesperetin inhibited aromatase expression at low concentration. We also illustrated that hesperetin in combination with letrozole did not exert any significant effect on aromatase.

Previous studies has shown that letrozole inhibit proliferation of MCF-7CA (MCF-7 transfected with CYP19), determined by cell-count assay, however similar to our results, no effect of letrozole was seen on wild-type MCF-7 cells proliferation (22, 23). In the study of Mitropoulou *et al.*, cell growth inhibition by letrozole was determined by measuring the number of cells



**Figure 3.** Aromatase expression ratio ( $\pm$ SE) compared with control after treatment of MCF-7 cells for 48 h with 100 nM testosterone without (control) or with different concentrations of letrozole (LET), hesperetin (HES) and both (LET+HES). \* $p$ -value=0.004 vs. control.



after stimulation with 4-androstene-3, 17-dione (AD) and testosterone (24), whereas in our study the viability of the cells was evaluated by the MTT assay without AD stimulating. Some studies have also indicated that letrozole can inhibit cell proliferation by blocking progression of cells through the cell cycle rather than by a direct cytotoxic effect (22).

The anti-proliferative effect of flavonoids was reported in breast cancers cell lines (13, 25, 26), but hesperetin showed no significant cytotoxic effects on MCF-7 and MDA-MB-231 cells (27). In the present study, we found that hesperetin at high concentration decreased viability of MCF-7 cells, which is consistent with previous studies (16). Hesperidin is able to decrease viability of human lymphoblastic cells in a dose- and time-dependent manner. This effect is attributed to the function of hesperidin as a ligand for Peroxisome Proliferator-Activated Receptor gamma (PPAR $\gamma$ ) which results in inhibition of cell proliferation and apoptosis. Inactivation of NF- $\kappa$ B in a PPAR $\gamma$ -independent manner has also been suggested as a mechanism for the anti-proliferative effect of hesperidin and its function as a chemo-sensitizing agent (28). Likewise, hesperetin has been shown to induce cell cycle arrest at the G1 phase, and apoptosis that leads to inhibition of cell proliferation (16).

Despite the evidences supporting the anti-proliferative property of hesperetin, its exact mechanism is not clearly understood.

It seems that the biological activities of flavonoids depend on their structure, and it is suggested that the number of hydroxyl groups in AC ring and the presence of the 2,3-double bond in C ring play an important role in cytotoxicity effect of flavonoids (27).

In the present study low but measurable levels of aromatase activity were detected in MCF-7 cells, which is in agreement with other reports (5, 29). Inhibition of aromatase enzyme with letrozole has been demonstrated in placental microsomal and breast cancers tissues (30), similar to our results. The inhibitory effects of some flavonoids have also been reported on *in vitro* models (6, 31-33). Previous studies have indicated the ability of phytochemicals (PCs) such as biochanin A, chrysin, naringenin, apigenin, genistein and quercetin to inhibit aromatase activities in mammary fibroblasts, MCF-7 cells and their co-cultures (34). Among the flavonoids tested, hesperetin has been shown to be effective in inhibition of aromatase in microsomes (18) and *in vivo* using an aromatase transfected MCF-7 breast cancer xenograft in ovariectomized mice (19), whereas aromatase activity was increased by hesperetin on *in vitro* models (8). In the present study, we found that although hesperetin slightly decreased aromatase activity, but the results were not significant. Probably, a 48 h treatment is too short to express significant effect of hesperetin.

It is noteworthy that most of the aromatase inhibition assays have been performed based on releasing microsomal tritiated water from different sources, most commonly from human placentas (18). In the present model system, enzyme activity was assessed based on E2 production from testosterone which is the substrate for the aromatase enzyme in breast cancer tissue (29). Inhibition of aromatase has been attributed to the interaction of the heme iron of CYP19 with the 4-oxo group on

the C ring of the flavonoid (33). However, some studies have reported that hesperetin is a less potent aromatase inhibitor than other flavonoid because it has a 3'-OH group and a methoxyl group instead of hydroxyl group at the 4' position (19).

In this study we demonstrated that CYP19 gene expression is detected at low but measurable amounts in wild-type MCF-7 cells. This is in agreement with other reports (6, 35). Letrozole decreases aromatase mRNA in MCF-7Ca (3) and MCF-7 cells that are co-cultured with stromal cells (36) and changes many classical estrogen dependent genes *in vivo* (37). In fact, letrozole specifically inhibit aromatase enzyme activity and total body aromatization by 99% (37). Information about the effect of hesperetin on aromatase expression is more limited. Our results demonstrated that hesperetin at low concentrations decreased aromatase expression. Similarly, previous reports have shown that some phytochemicals reduced aromatase mRNA expression (6, 8, 31). In contrast, Li and coworkers reported that hesperetin treatment increased the expression of aromatase at or above 10  $\mu$ M for 24 h and induced the transactivity of promoters I.3 and II and transcriptional factors (8). It is still unclear whether the hydroxyl group at 3' and 4' is responsible for the induction of expression, or the metabolites of flavonoids might regulate transcription. Some flavonoid such as luteolin suppressed the aromatase expression via signaling pathway (8). These differences point out the necessity for further investigations.

In the treatment of breast cancer, chemotherapy may lead to better results compared with single chemotherapeutic agents and may increase the efficacy of chemotherapeutic agents (38).

Inhibition of aromatase mRNA expression has been observed after 48 h exposure of human granulose-luteal cells to a combination of isoflavones (39). This inconsistency with our results may be due to different cell line and flavonoids.

The strength of the present study is that the investigated concentrations are achievable *in vivo*, based on little information in animal and human study (8, 19, 40); whereas most of the experimental studies have been performed with flavonoids concentrations more than usual dietary intake.

In conclusion, our study showed that hesperetin could decrease aromatase expression at low concentration in MCF-7 breast cancer cells. Since, hesperetin could be effective in reduction of aromatase expression; it is probable that continuous exposure has more profound effects. Hence further studies using animal models are required to identify the beneficial effect of hesperetin on aromatase transcription in breast cancer.

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