Original Research

The effects of Nobiletin, Hesperetin, and Letrozole in a combination on the activity and expression of aromatase in breast cancer cells

S. T. Rahideh[1], M. Keramatipour[2], M. Nourbakhsh[3], F. Koohdani[4], M. Hoseini[5], F. Shidfar[1*]

[1] Department of Nutrition, School of Public Health, Iran University of Medical Sciences, Tehran, Iran
[2] Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[3] Department of Biochemistry, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[4] Department of Molecular and Cellular Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran
[5] Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Abstract: Nobiletin (NOB) and hesperetin (HES) are the citrus polymethoxyflavone and flavonone. Aromatase or cytochrome P450 (CYP19) enzyme is a key enzyme in estrogen biosynthesis. The objective of this study was to investigate the combinational effects of HES, NOB and letrozole (LET) as aromatase inhibitors on the activity and expression of aromatase in MCF-7 cells. In this study, aromatase enzyme activity based on the conversion of androgen substrate testosterone to 17β-Estradiol was determined. Estradiol concentrations were measured using an electrochemiluminescence immunoassay. CYP19 gene expression was determined by quantitative real-time PCR. Our findings demonstrated that none of combinations including LET+NOB, LET+HES, LET+NOB+HES, and NOB+HES had no significant effects on aromatase activity and expression. The present study showed for the first time that the combination of HES, NOB, and LET had no effects on activity and expression of aromatase in MCF-7 breast cancer cells.

Key words: Nobiletin; Hesperetin; Letrozole; Aromatase; Breast cancer; MCF-7 cells.

Introduction

According to WHO reports, cancer is currently the leading cause of death and prevails coronary heart disease or stroke (1). Among Iranian women, breast cancer is a commonly diagnosed malignancy and the most frequent cause of cancer death, accounting for 23% of all cancers and 14% of the cancer deaths (2, 3).

Approximately 80% of breast cancer cases are estrogen receptor positive; thus endocrine treatment is of one major therapeutic value in these patients along with surgery (4). 17β-Estradiol (E2) plays an important role in the pathogenesis and progression of breast cancer (5, 6). Estrogen biosynthesis requires the key enzyme aromatase (7). Aromatase is a member of the cytochrome P450 (CYP 19) enzyme family, which converts androgens such as testosterone and androstenedione to estrogens such as 17β-estradiol and estrone (8). Activity and expression of aromatase as well as plasma estrogen concentrations increase in breast cancer tissue (6, 9, 10). Hence, aromatase inhibitors (AIs) are being used clinically in the estrogen-responsive breast cancer treatment (4, 8). Letrozole (LET) is a useful non-steroidal AIs used as first-line therapy in the treatment of estrogen receptor positive breast cancer in postmenopausal women (4, 6, 7). Several studies have demonstrated the superiority of the third-generation AIs for example letrozole to tamoxifen, which is a selective estrogen receptor. These findings confirm that adjuvant therapy with AIs reduces the recurrence risk in patients with advanced breast cancer (4, 11-13). The AIs response rate is also improved when compared with tamoxifen in postmenopausal women (14). A new class of AIs like LET has been classified as non-steroidal aromatase inhibitors (NSAIs), consists of natural products and their derivatives such as flavonoids (7).

High intake of citrus fruits is associated with a 10% decrease in breast cancer risk in women (15).

The chemopreventive potential of polyphenolic compounds in the recent years has clearly supported their health benefits, including anti-cancer properties. The effects of dietary polyphenols on aromatase may provide an effective strategy for reducing the health burden of cancer in humans (16).

Flavonoids are the most important group of phenolic compounds in plants (17) due to their low toxicity and effectiveness in chemopreventive therapy (8, 18). Flavonoids in citrus have shown a wide range of chemopreventive properties and exhibited anti-proliferative activities toward the human cell lines in numerous in vitro studies (19-21). Nobiletin (NOB) and hesperetin (HES) are the citrus polymethoxyflavone (PMFs) and flavonone which have shown a wide range of biological activities, including anti-inflammatory, anti-carcinogenic, anti-viral, antioxidant, anti-thrombogenic, and anti-artherogenic properties (18, 22).

Combined chemotherapy in the treatment of breast cancer may increase chemotherapeutic agents' efficacy,

resulting in decreased toxicity for normal tissues (15). Citrus flavonoids increased their cytotoxic effects, modulated cell cycle and induced apoptosis of cancer cells when they were used in a combination with chemotherapeutic agents (15).

To date, various types of flavonoids have been investigated for aromatase inhibition using a microsomal assay (23). However, few studies have assessed the effect of citrus flavonoids on aromatase expression (9). Inhibition of the CYP 19 expression has been reported after exposure to a combination of isoflavones and flavones in human granulose-luteal cells (24).

Since LET is mostly prescribed for patients with breast cancer, assessing other AIs in a combination with LET would be of great value. Nevertheless, information about the possible ability of flavonoids and AIs in a combination is even more limited and until now the effect of citrus flavonoids and AIs in a combination on aromatase has not been examined. Therefore, the present study was carried out to investigate the effect of combinations of HES, NOB and LET on the activity and expression of aromatase in MCF-7 breast cancer cells.

Materials and Methods

Human breast cancer MCF-7 cells (Avicenna Research Institute, Iran) were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, USA), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco BRL, Grand Island, NY, USA), 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA). The samples were incubated at 37°C in a humidified incubator with 5% CO_2 atmosphere. The cells were routinely subcultured when 80% confluence was reached. Then, the cells were trypsinized using trypsin-EDTA (Gibco BRL, Grand Island, NY, USA). The cells were seeded into 6-well plates at a density of 3×10^5 cells/well for aromatase activity and expression assays and were treated with nobiletin (Sigma-Aldrich, Germany), hesperetin (Sigma-Aldrich, Germany) and letrozole (Ind-Swift-India, India). All compounds were dissolved in dimethyl sulfoxide (DMSO) (Applichem, USA) with final DMSO concentrations less than 0.1% in culture media. The combined groups consisted of letrozole and nobiletin (LET+NOB); letrozole and hesperetin (LET+HES); letrozole, nobiletin, and hesperetin (LET+NOB+HES); nobiletin and hesperetin (NOB+HES). All compounds were added to the culture medium at final concentrations of 1 μM for LET and NOB and 5 μM for HES. Each experiment was performed in duplicate and repeated three times.

Aromatase activity assay

The activity of aromatase enzyme was assessed by measuring the conversion of androgen substrate testosterone to E_2 in cell culture (25). Briefly, MCF-7 cells (3×10^5 cells/well) were seeded in the medium supplemented with 5% FBS. Culture medium was removed after one day and replaced by phenol red-free RPMI medium (Biosera, UK) containing 2% dextran-coated charcoal-stripped (Sigma Chemical Co, St Louis, MO, USA) FBS (DCC-FBS). Testosterone with 100 nM concentration (Sigma Chemical Co, St Louis, MO, USA) was also added as aromatase substrate and incu-

bated overnight. The cells were treated with various combinations for 48 h. Subsequently, the media were collected and stored at –70°C until further analysis. E2 concentrations were measured using an electrochemiluminescence immunoassay (ECLIA) kit (Roche, Germany) by elecsys 2010 instrument with sensitivity of 18.4 pmol/L, an intra-assay coefficient of variation (CV) of 1.9–5.7% and an inter-assay CV of 2.3–6.2%.

Protein concentrations were determined by the Bradford method (26). Aromatase activity was calculated as picograms of E2 synthesized per milligram protein per hour (pg E2/mg protein/ hour) and expressed as percentage of control.

RNA extraction and cDNA synthesis

Total RNA was isolated from the cells using a Ribospin kit (GeneAll, South Korea) according to manufacturer's instructions and stored at -70°C until analysis. RNA quantity and quality were assessed by measuring absorption in 260 nm and 280 nm using Nano Drop spectrophotometer (ND-2000c) (Wilmington, Delaware USA) and by gel electrophoresis, respectively. Complementary DNA (cDNA) was synthesized using a Hyper-Script™ RT master mix (GeneAll, South Korea) with 1 μg of total RNA in a 20 μl reaction mixture using oligo dT and randomhexamer primers. The reverse transcription (RT) PCR was initiated by pre-heating the mixture 5 min at 65 °C to facilitate the reaction by denaturing the secondary structure of RNA, followed by adding master mix solution. The reaction was performed for 5 min at 25 °C, followed by 60 min at 55 °C. The reaction was terminated at 95 °C for 5 min and chilled on ice. cDNA stored at −20°C prior to real-time PCR assay.

Quantitative real-time PCR assay of CYP19 expression

Real-time PCR was performed by a Corbet rotor gene real-time PCR system, utilizing SYBR Green reagents (Ampliqon, Denmark). Primer sequences were as follows: forward and reverse- CYP19 (5′-GAATTG-GACCCCTCATCTCCC-3′ and 5′-GCCGAATCGA-GAGCTGTAAT-3′); Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalizer (5'-GGG-TGTGAACCATGAGAAGT-3' and 5'-GGCATGGAC-TGTGGTCATGA-3'). These primers were obtained from Macrogen Company (South Korea). Amplification reactions were run as triplicates. Each reaction was performed in a 20 μl total volume mixture containing 10 μl of SYBR Green reagents, 200 nmol/L of forward and reverse primers and 1 μl of cDNA sample. The reaction was initiated by heating at 95°C for 15 min, followed by 45 amplification cycles of 95°C for 15 s, 62°C for 20 s, and 72°C for 30 s. Melt curves were acquired to ensure the specificity of amplification, after each real time PCR. PCR products were also confirmed by gel electrophoresis.

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 the expression ratio:

$$\text{Expression ratio} = \frac{(E_{target})^{\Delta Ct_{targrt}}}{(E_{reference})^{\Delta Ct_{reference}}} \tag{1}$$

Statistical analysis

Each experiment was repeated at least three times, and each treatment was tested in duplicate. Values were expressed as means ± standard error (SE). Statistical significance of differences between samples was 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 values of < 0.05 were considered to be significant.

Results

Aromatase enzyme activity

Aromatase enzyme activity was determined based on converting of testosterone to E2 after 48 h treatments with a combination of NOB, HES and LET.

Figure1 shows the effect of NOB, HES, LET in a combination on the activity of aromatase. In comparison with control, all groups did not have any significant effect on aromatase activity (Figure 1). So, the combination of these compounds did not significantly alter the mean differences of E2 concentration before and after treatment between groups.

CYP 19 gene expression

Expression of CYP19 gene was determined using quantitative real-time PCR.

No significant changes in the CYP19 gene expression were observed upon exposure to combination of compounds compared with control (Figure 2).

Discussion

Despite the efficacy of AIs or other endocrine therapies for the treatment of breast cancer, patients acquire resistance to AIs that consequently cause a challenge in disease management and facing many problems (23, 27). Synthetic AIs show serious side effects, which is generally related to estrogen deprivation. The quality of life side effects are also often seen with the use of AIs. Developing a resistance to AIs results in a relapse of the disease in postmenopausal breast cancer patients. Several mechanisms have developed to overcome these side effects such as the use of combination therapies with
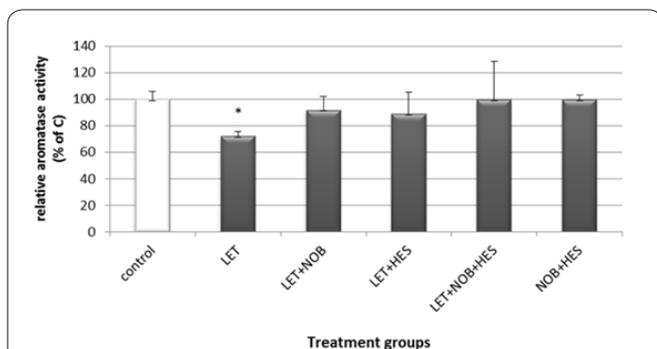
Figure 1. Aromatase activity as the percentage of control (±SE) after incubation of MCF-7 cells for 48 h with 100 nM testosterone without (control) or with letrozole and nobiletin (LET+NOB); letrozole and hesperetin (LET+HES); letrozole, nobiletin, and hesperetin (LET+NOB+HES); nobiletin and hesperetin (NOB+HES). The results are the mean ± SE of duplicate experiments that were repeated at least three times. * *P* < 0.05 vs. control.
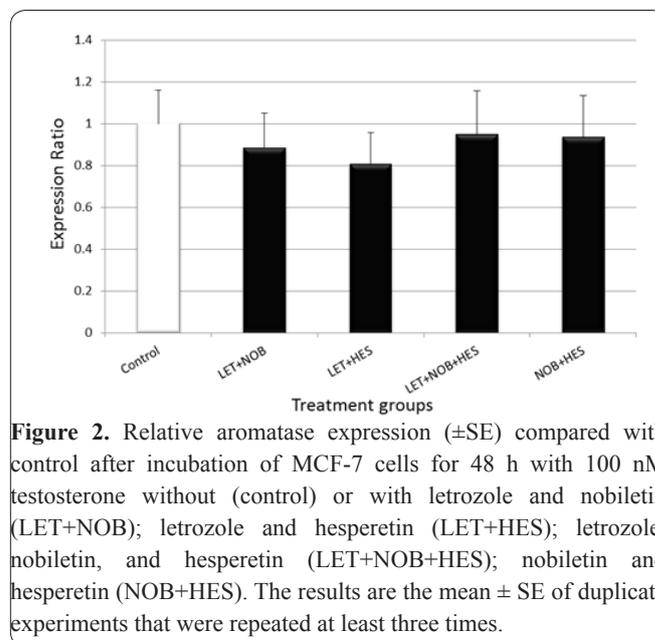
Figure 2. Relative aromatase expression (±SE) compared with control after incubation of MCF-7 cells for 48 h with 100 nM testosterone without (control) or with letrozole and nobiletin (LET+NOB); letrozole and hesperetin (LET+HES); letrozole, nobiletin, and hesperetin (LET+NOB+HES); nobiletin and hesperetin (NOB+HES). The results are the mean ± SE of duplicate experiments that were repeated at least three times.

AIs and other compounds like PCs (23).

Therefore, therapeutic agents that can be used for a treatment of breast cancer and/or enhance sensitivity to AIs would be of great importance (27). Moreover, combined with a chemopreventive agent can increase clinical efficacy and less severe side effects of chemotherapeutic agents and overcome the problem of resistance in cancer cells (18, 23). Thus, co-chemotherapy may lead to better results (15). HES could improve doxorubicin cytotoxic chemotherapy (28). In combination, nobiletin synergistically increases doxorubicin's cytotoxic activity (15). Flavonoids in combination with AIs could offer reduced drug toxicity to normal tissue and increased effectiveness of AIs (18, 23).

In the present study, MCF-7 cells were observed to express sufficient aromatase activity based on E2 production from testosterone, which is the substrate for the enzyme in breast cancer tissue. This is in agreement with other reports (6, 29). LET as an AI specifically inhibits aromatase enzyme activity and total body aromatization by 99% (30). Previous studies have also reported the ability of some phytochemicals (PCs) such as biochanin A, chrysin, naringenin, apigenin, genistein and quercetin to inhibit aromatase in placental microsomes and breast cancer tissues (10, 31-34). It has been shown HES is able to inhibit aromatase activity in microsomes (23) and in vivo (35). In our previous study, we revealed that NOB at low concentration decreased aromatase activity (36). Similar to the present studies, a combination of some PCs didn't inhibit aromatase in short incubation time (24). However, the combinational effects of HES, NOB, and LET on aromatase activity have not been studied before. In the present study, the combination of NOB, HES and LET had no effect on aromatase. Consistently, Rice *et al* showed that the combination of isoflavones and flavones did not inhibit aromatase activity after 48 h exposure, although prolonged exposure for120 h inhibited aromatase activity (24). This long term effect suggests an indirect mechanism. In our experiment, the cells were treated for 48 h. Longer incubation time might have been effective on aromatase activity, however, we looked for a direct effect and therefore did not extend the incubation time.

In our previous in vitro study, we demonstrated that HES and NOB at low concentration are inhibitors of CYP19 gene expression (36, 37). Inhibition of the CYP 19 expression has also been observed after 48 h treatment of human granulose-luteal cells with a combination of isoflavones and flavones (genistein, daidzein, and biochanin A) (24). Nevertheless, different cell lines and flavonoids may have resulted in dissimilar outcomes compared to ours. In addition, cell culture conditions are also known to influence gene expression levels (34). In the present study, the combination of LET, HES, NOB did not significantly alter aromatase expression, however, changes in the activity of aromatase and its gene expression followed the same trend.

Moreover, we showed that the inhibitory function of LET was compromised in the presence of HES and NOB. Interactions of flavonoids with cytochromes play an important role in drug metabolism. Flavonoids might induce cytochromes and/or modulate their activity (38). Since letrozole is metabolized mainly by the cytochromes P450 (39), its lack of function in the presence of flavonoids may be the result of its augmented metabolism. Thus, these drugs–flavonoid interactions are suggested to be carefully examined in future studies.

Despite the little information on animal and human study, we investigated the effect of achievable concentrations in vivo (9, 35, 40). While most of the experimental studies have been performed with flavonoids concentrations exceeding usual dietary intake. On the other hand, we could not perform aromatase western analyses to determine the effect, but it could be recommended in future works.

To our knowledge, this is the first time to show that HES, NOB and LET in a combination that has no effect on aromatase activity and expression in MCF-7 breast cancer cells. The lack of any significant inhibition of aromatase suggests that continuous exposure may have more profound effects. Further studies including animal models need to be investigated to identify the effect of NOB, HES and LET in a combination on aromatase transcription in breast cancer.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer 2015;136 (5): E359-E86.

2. Mousavi SM, Gouya MM, Ramazani R, Davanlou M, Hajsadeghi N, Seddighi Z. Cancer incidence and mortality in Iran. Annals of Oncology 2008:mdn642.

3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians 2011;61(2):69-90.

4. Lumachi F, Santeufemia DA, Basso SM. Current medical treatment of estrogen receptor-positive breast cancer. World journal of biological chemistry 2015;6(3):231.

5. Chen D, Reierstad S, Lu M, Lin Z, Ishikawa H, Bulun SE. Regulation of breast cancer-associated aromatase promoters. Cancer letters 2009;273(1):15-27.

6. Brooks JD, Thompson LU. Mammalian lignans and genistein decrease the activities of aromatase and 17β-hydroxysteroid dehydrogenase in MCF-7 cells. The Journal of steroid biochemistry and molecular biology 2005;94(5):461-7.

7. Bonfield K, Amato E, Bankemper T, Agard H, Steller J, Keeler JM, et al. Development of a new class of aromatase inhibitors: Design, synthesis and inhibitory activity of 3-phenylchroman-4-one (isoflavanone) derivatives. Bioorganic & medicinal chemistry 2012;20(8):2603-13.

8. Khan SI, Zhao J, Khan IA, Walker LA, Dasmahapatra AK. Potential utility of natural products as regulators of breast cancer-associated aromatase promoters. Reprod Biol Endocrinol 2011;9:91.

9. Li F, Ye L, Lin S-m, Leung LK. Dietary flavones and flavonones display differential effects on aromatase (CYP19) transcription in the breast cancer cells MCF-7. Molecular and cellular endocrinology 2011;344(1):51-8.

10. Ye L, Gho WM, Chan FL, Chen S, Leung LK. Dietary administration of the licorice flavonoid isoliquiritigenin deters the growth of MCF-7 cells overexpressing aromatase. International Journal of Cancer 2009;124(5):1028-36.

11. Mauri D, Pavlidis N, Polyzos NP, Ioannidis JP. Survival with aromatase inhibitors and inactivators versus standard hormonal therapy in advanced breast cancer: meta-analysis. Journal of the National Cancer Institute 2006;98(18):1285-91.

12. Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. Journal of Clinical Oncology 2010;28(3):509-18.

13. van Duursen MB, Nijmeijer S, de Morree E, de Jong PC, van den Berg M. Genistein induces breast cancer-associated aromatase and stimulates estrogen-dependent tumor cell growth in in vitro breast cancer model. Toxicology 2011;289(2):67-73.

14. Lønning P. The potency and clinical efficacy of aromatase inhibitors across the breast cancer continuum. Annals of Oncology 2011;22(3):503-14.

15. Wang L, Wang J, Fang L, Zheng Z, Zhi D, Wang S, et al. Anticancer activities of citrus peel polymethoxyflavones related to angiogenesis and others. BioMed research international 2014;2014.

16. Link A, Balaguer F, Goel A. Cancer chemoprevention by dietary polyphenols: promising role for epigenetics. Biochemical pharmacology 2010;80(12):1771-92.

17. Holick M, Shils M, Shike M, Ross A, Caballero B, Cousins R. Modern nutrition in health and disease. 2006. In: Shils, OJME [Internet]. [329-45].

18. Meiyanto E, Hermawan A, Anindyajati A. Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. Asian Pacific Journal of Cancer Prevention 2012;13(2):427-36.

19. Camarda L, Di Stefano V, Del Bosco SF, Schillaci D. Antiproliferative activity of Citrus juices and HPLC evaluation of their flavonoid composition. Fitoterapia 2007;78(6):426-9.

20. Manthey JA, Guthrie N. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. Journal of Agricultural and Food Chemistry 2002;50(21):5837-43.

21. Luo G, Guan X, Zhou L. Apoptotic effect of citrus fruit extract nobiletin on lung cancer cell line A549 in vitro and in vivo. Cancer biology & therapy 2008;7(6):966-73.

22. Xiao H, Yang CS, Li S, Jin H, Ho C-T, Patel T. Research Article Monodemethylated polymethoxyflavones from sweet orange (Citrus sinensis) peel Inhibit growth of human lung cancer cells by apoptosis. Mol Nutr Food Res 2009;53:000-.

23. Balunas MJ, Su B, Brueggemeier RW, Kinghorn AD. Natural products as aromatase inhibitors. Anti-cancer agents in medicinal

chemistry 2008;8(6):646.

24. Rice S, Mason HD, Whitehead SA. Phytoestrogens and their low dose combinations inhibit mRNA expression and activity of aromatase in human granulosa-luteal cells. The Journal of steroid biochemistry and molecular biology 2006;101(4):216-25.

25. Krishnan AV, Swami S, Peng L, Wang J, Moreno J, Feldman D. Tissue-selective regulation of aromatase expression by calcitriol: implications for breast cancer therapy. Endocrinology 2010;151(1):32-42.

26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 1976;72(1):248-54.

27. Long X, Fan M, Bigsby RM, Nephew KP. Apigenin inhibits antiestrogen-resistant breast cancer cell growth through estrogen receptor-α-dependent and estrogen receptor-α-independent mechanisms. Molecular cancer therapeutics 2008;7(7):2096-108.

28. Marcsek Z, Kocsis Z, Jakab M, Szende B, Tompa A. The efficacy of tamoxifen in estrogen receptor-positive breast cancer cells is enhanced by a medical nutriment. Cancer biotherapy & radiopharmaceuticals 2004;19(6):746-53.

29. Sonne-Hansen K, Lykkesfeldt A. Endogenous aromatization of testosterone results in growth stimulation of the human MCF-7 breast cancer cell line. The Journal of steroid biochemistry and molecular biology 2005;93(1):25-34.

30. Mackay A, Urruticoechea A, Dixon JM, Dexter T, Fenwick K, Ashworth A, et al. Molecular response to aromatase inhibitor treatment in primary breast cancer. Breast Cancer Res 2007;9(3):R37.

31. Wang Y, Man Gho W, Chan FL, Chen S, Leung LK. The red clover (Trifolium pratense) isoflavone biochanin A inhibits aromatase activity and expression. British Journal of Nutrition 2008;99(02):303-10.

32. Monteiro R, Faria A, Azevedo I, Calhau C. Modulation of breast cancer cell survival by aromatase inhibiting hop (Humulus lupulus L.) flavonoids. The Journal of steroid biochemistry and molecular biology 2007;105(1):124-30.

33. Sanderson JT, Hordijk J, Denison MS, Springsteel MF, Nantz MH, Van Den Berg M. Induction and inhibition of aromatase (CYP19) activity by natural and synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells. Toxicological Sciences 2004;82(1):70-9.

34. Van Meeuwen J, Korthagen N, De Jong P, Piersma A, Van den Berg M. (Anti) estrogenic effects of phytochemicals on human primary mammary fibroblasts, MCF-7 cells and their co-culture. Toxicology and applied pharmacology 2007;221(3):372-83.

35. Ye L, Chan FL, Chen S, Leung LK. The citrus flavonone hesperetin inhibits growth of aromatase-expressing MCF-7 tumor in ovariectomized athymic mice. The Journal of nutritional biochemistry 2012;23(10):1230-7.

36. Rahideh ST, Keramatipour M, Nourbakhsh M, Koohdani F, Hoseini M, Talebi S, Shidfar F. Comparison of the effects of Nobiletin and Letrozole on the activity and expression of aromatase in MCF-7 breast cancer cell line. Biochem Cell Biol. 2017 Feb 1. doi: 10.1139/bcb-2016-0206. [Epub ahead of print]

37. Rahideh ST, Shidfar F, Nourbakhsh M, Hoseini M, Koohdani F, Entezam M, et al. The individual or combinational effects of Hesperetin and Letrozole on the activity and expression of aromatase in MCF-7 cells. Cellular and molecular biology (Noisy-le-Grand, France) 2016;62(6):38-43.

38. Hodek P, Trefil P, Stiborová M. Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. Chemico-biological interactions 2002;139(1):1-21.

39. Desta Z, Kreutz Y, Nguyen A, Li L, Skaar T, Kamdem L, et al. Plasma letrozole concentrations in postmenopausal women with breast cancer are associated with CYP2A6 genetic variants, body mass index, and age. Clinical Pharmacology & Therapeutics 2011;90(5):693-700.

40. Kanaze F, Bounartzi M, Georgarakis M, Niopas I. Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. European journal of clinical nutrition 2007;61(4):472-7.