

The Effect of Myricetin Flavonoid on the Expression of Fyn Gene in Melanoma Cells (A375)

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Abstract

Background: Malignant melanoma as one of the most common cancers is currently spreading worldwide. Regarding after-effect of advanced treatments, using natural products has attracted much attention. Flavonoids, polyphenol compounds rich in diet, are being considered for their therapeutic preventive features. Fyn gene, a member of the protein tyrosine kinase oncogene family, has become an important target for therapy goals.

Objectives: The aim of this study was to assess Fyn gene expression after treatment of melanoma cells with myricetin.

Methods: In this study, the melanoma cells were treated with different concentrations of myricetin (0 to 100 μM) and their viability was determined by the methylthiazolyl diphenyl-tetrazolium bromide (MTT) assay, also the expression of Fyn gene in treated cells with selected concentrations of myricetin (0, 20, 40, 50, and 60 μM) was detected by real time quantitative polymerase chain reaction (qPCR).

Results: The current investigation showed that treatment of A375 melanoma cells with the dietary flavonoid myricetin (3, 5, 7-trihydroxy-2-(3, 4, 5-trihydroxy phenyl)-4-chromenone), resulted in decreased cell viability and increased expression of Fyn gene. The MTT assay analysis of exposed cells with different concentrations of myricetin showed that up to 25 μM of myricetin had no cytotoxicity effect on A375 cells, also with increasing of myricetin concentration, the repression of cell proliferation developed as well.

Conclusions: Real time qPCR analysis of Fyn expression in exposed cells with various concentration of myricetin leads to overexpression of this gene, dose dependently. Through this research, it was determined that myricetin with its anti-proliferative potential could suppress the development of cancer cells. On the other hand, since Fyn kinase could be involved in tumorigenesis of some cancer cells, it could be concluded that myricetin could effect the carcinogenicity of Fyn function in melanoma cells.

Keywords: Melanoma, A375, Myricetin, Fyn Gene

1. Background

Cancer, as a leading cause of death globally, has been on the spotlight for many researchers. According to the American cancer society, melanoma is considered among the most common cancers worldwide and regarding the adaption of new lifestyle, the occurrence of malignant melanoma is increasing, leading to high mortality rates (1).

In the recent decades, due to harmful health effect of usual cancer treatment, many researches have been done to constitute natural substances for medicine purposes. Flavonoids, natural compound with low molecular weight, on account of pharmacological properties, such as anticancer, antioxidant, anti-inflammatory, and anti-angiogenesis activities, are considered as one of the most interesting subjects in many studies (2). A basic c6-c3-c6 phenyl-benzopyran backbone is shared in all flavonoids and different groups of flavonoids, based on the position of B ring relative to C ring, C2-C3 double bound, and 3-OH

groups, have been classified (3) (Figure 1) (4).

Among these natural products, myricetin is one of the best compounds with inhibitory and anti-oxidant abilities (Figure 2) (5).

Myricetin, present in different kinds of fruits, could modulate the activity of important signaling molecules, such as protein kinase (ERK1/2) in lung cancer cells (6) and kinase B (Akt) in cervical and lung cancer cell lines (7, 8). These pathways could influence growth and survival of cancer cells, and result in generation and cell cycle progression.

Some transcriptional factors, like NF- κ B, AP-1 and STAT3, are key factors in cell transduction, the function of which is inhibited by myricetin (9, 10).

Two classes of tyrosine kinases (TKs), important classes of molecules in human biology are receptor and non-receptor TKs. Receptor TKs, involving epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and mesenchymal epithelial transition

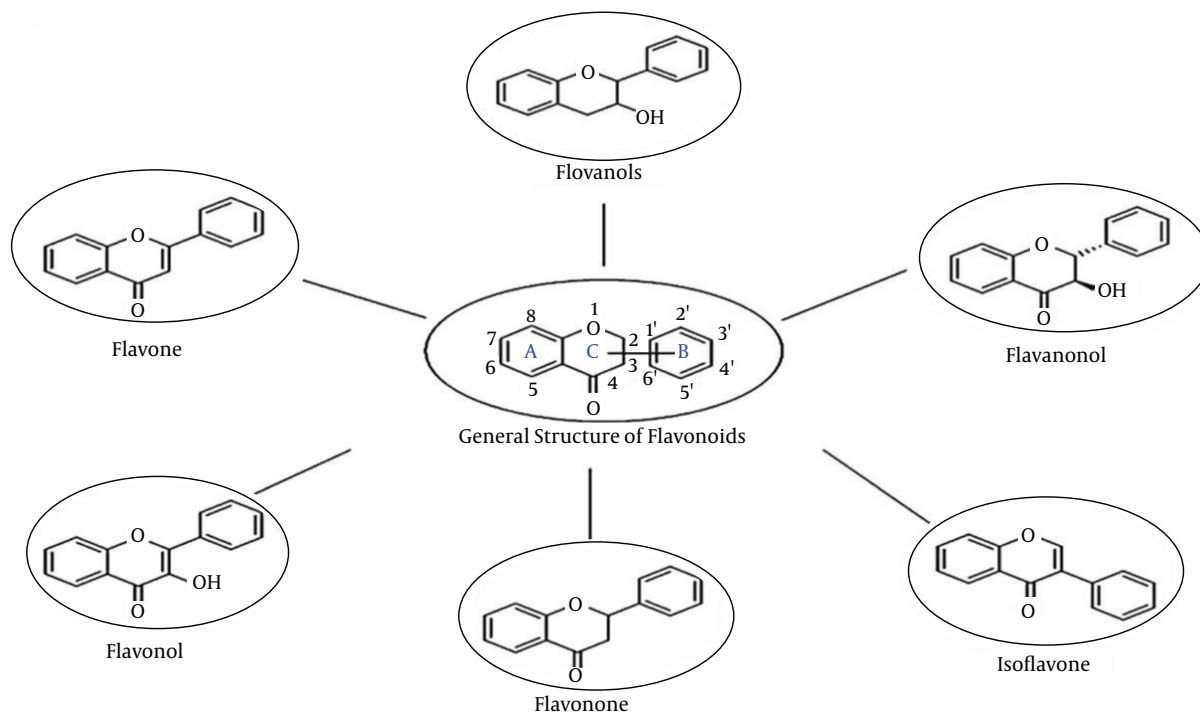


Figure 1. Classification of Flavonoids

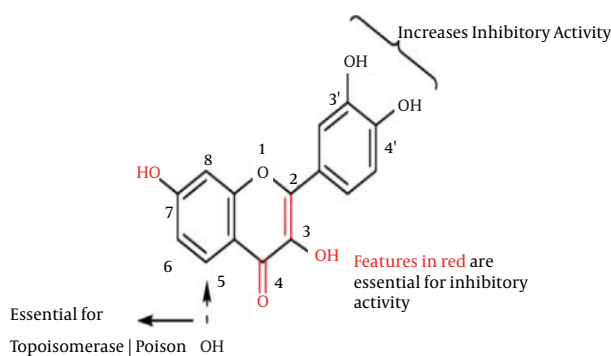


Figure 2. Myricetin Structure

factor (c-MET), are membrane proteins receiving signals from ligands. Non receptor TKs include members of families, such as Abl, Src, and focal adhesion kinase that by being activated, could stimulate downstream signals to process growth and motility of cancer cells, the activation of which is firmly organized (11).

The Src families participate in diverse signaling processes, involving mitogenesis, T- and B-cell activation in addition to being shared in several human carcinomas, such

as breast, lung, and colon cancer (12).

Fyn, a member of the Src family, could phosphorylate tyrosine residues of targets in different signaling pathway, overexpressed in a variety of cancers, including glioblastoma multiform, squamous cell carcinoma of the head and neck, and prostate cancer (13). There is a relationship between the inhibition of Fyn and decreased cell growth. In the PI3K/Akt/PKB pathway, Fyn acts as a mediator of growth factor, and induces the anti-apoptotic activity of Akt/PKB (14). Some studies determined that Fyn overexpression promotes anti-apoptotic activity of Akt. On the other hand, activation of Akt in some cancers, such as prostate cancer, is common. Further studies are required to elucidate the mechanism of Fyn function and dysregulation of Akt activity (15).

Also, it has been reported that Fyn has a role in epithelial-mesenchymal transition (EMT) process, including loss of cell adhesion, increase of cell motility, and change from E-cadherin to N-cadherin expression (16).

2. Methods

2.1. Reagents

Myricetin and methylthiazolyl diphenyl-tetrazolium bromide (MTT) powder were purchased from sigma

Aldrich. The A375 cell line was prepared from the pasture institution. Fetal bovine serum (FBS), phosphate-buffered saline (PBS) tablet, penicillin-streptomycin, trypsin-EDTA, and first strand cDNA synthesis were obtained from Invitrogen. The RNA extraction kit was prepared from gene all. Furthermore, qPCR Green Master with low ROX was purchased from jena bioscience. Primers were prepared from Takapouzist. Culture Media (DMEM high glucose) were obtained from biosera.

2.2. Cell Culture

A375 cells were cultured in DMEM high glucose, containing 10% (v/v) FBS and 1% penicillin-streptomycin. The cells were incubated at 37° in a humidified atmosphere of 5% CO₂. Confluent cultures of melanoma cells were rinsed in phosphate-buffered saline (PBS) and removed from the flask using 0.25% trypsin/EDTA, neutralized with FBS and the cell suspension was prepared.

2.3. Cell Counting

To find the doubling time of melanoma cells, 8000 cells were placed on each well in a 96-well plate. During one week at different periods of time, the cells were counted and the data were recorded (17).

2.4. Determination of Cell Viability

Cell viability was detected by using the micro culture tetrazolium technique (MTT). This quantitative measure provides the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated versus control cells (17).

2.5. Treatment of Cells with Myricetin

For RNA extraction, 3×10^5 cells were seeded in 6-well plates and were exposed to various concentrations of myricetin (control, 0.1% Ethanol, 20, 40, 50, and 60 μM) for 48 hours. The concentrations were chosen based on acquired IC₅₀ from MTT results. The experiments were done in triplicates.

2.6. Real Time qPCR

Real-time qPCR was used to determine the expression of Fyn gene after 48 hours of treatment. Total RNA was extracted using Trizol (Gene All), according to the manufacturer's instructions, and then quantified. Its concentration was detected spectrophotometrically at 260 nm (Nanodrop 1000 UV-Vis Spectrophotometer). First strand complementary DNAs (cDNA) were synthesized from 5 ng of the isolated RNA by random Hexamer primer using RevertAid first strand cDNA synthesis kit (Fermentase), and used as templates for real time qPCR. The expression of

mRNAs was determined quantitatively using qPCR green master with lowROX kit. The PCR was performed on a final volume of 20 μL , containing 1 μL of cDNA, 250 nM of each primer, and 10 μL of qPCR Green Master, with ABI 7500 real time step one plus system (Applied Biosystems, USA). The primers used for Eva green real time qPCR were as follows: Fyn Forward: 5'-ATTGCTGACTTCGGATTGGC-3', Fyn Reverse: 5'-TGGCACTCTTCCTTTGGTGA-3', GAPDH Forward: 5'-CAATGACCCCTTCATTGACC-3', and GAPDH Reverse: 5'-TGGAAGATGGTGATGGGATT-3'.

The samples underwent 40 cycles consisting of the following steps: initial denaturation at 95°C for 2 minutes, followed by a set cycle of denaturation at 95°C for 15 seconds, annealing and elongation temperature at 60°C for 1 minute. Fold increment of gene, based on the pfaffl method, at different concentrations was calculated by normalizing its expression level to that of the glyceraldehyde 3-Phosphate dehydrogenase (GAPDH) gene, which was used as an internal control. The formula was as below:

$$\text{Fold difference} = (\text{E target})^{\Delta\text{Ct target}} / (\text{E normalizer})^{\Delta\text{Ct normalizer}}$$

$$\text{E} = \text{efficiency from standard curve } E = 10^{-1/\text{slope}}$$

$$\Delta\text{Ct target} = \text{Ct GOI c} - \text{Ct GOI s}$$

$$\Delta\text{Ct normalizer} = \text{Ct norm c} - \text{Ct norm s. (18)}$$

2.7. Statistical Analysis

All experiments reported in this study were accomplished independently 3 times. Results are expressed as mean \pm standard deviation (SD). Statistical analyses of the significance of differences among values were carried out by one-way analysis of variance (ANOVA), using the PRISM software. Values of $P < 0.05$ were considered statistically significant.

2.8. Ethical Issue

This study was in accordance with the Helsinki declaration, approved by the regional institution number: IR.QUMS.REC.1394.173 Qazvin University of medical science.

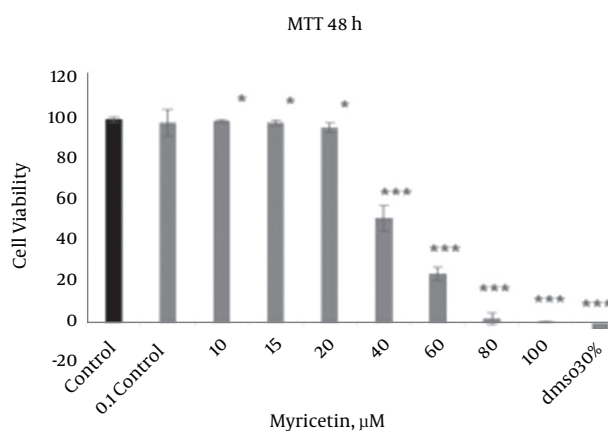
3. Results

3.1. Doubling Time Distribution

The results of counting melanoma cells (A375) acknowledged that the doubling time of cells was 17 hours (17).

3.2. The MTT Assay

To ascertain the inhibitory effect of myricetin on melanoma cells, A375 cells were treated with different concentrations (0 to 100 μM) for 48 hours. Due to the current findings, with increasing concentration of myricetin, cell proliferation was reduced. On the other hand, the IC₅₀ of myricetin for 48 hours was 40 μM (Figure 3) (17).

Figure 3. Myricetin Inhibition of Proliferation of Melanoma Cells After 48 Hours

Data are presented as the mean \pm SD of the 5 experiments (* $P < 0.05$, *** $P < 0.0001$) versus the control group.

3.3. Treatment of Cells with Myricetin

As shown in [Figure 4](#), 48 hours of exposure of cells to myricetin resulted in reduction of cell proliferation, dose dependently.

3.4. The mRNA Expression of Fyn

Real time qPCR was used to detect the expression of Fyn gene when melanoma cells (A375) were cultured in DMEM medium supplemented with different concentrations of myricetin for 48 hours. The mRNA expression of Fyn was increased in the treated group, dose dependently, with the results being significant compared to the 0.1% Ethanol group ($P < 0.05$) ([Figure 5](#)).

4. Discussion

Myricetin, rich in fruits and vegetables, is identified to have different biological influence with multiple mechanisms. In this study, the authors determined that myricetin has an antiproliferative effect on melanoma cells so that it could suppress the growth of melanoma cells, and increase the expression of Fyn mRNA in these cells, which had been decreased previously (before treatment as a sign of advanced cancer).

In the last few years, considerable advancement has been made in melanoma treatment, however, the prosperous therapy is still a challenge. The activation of PI3K/Akt signaling pathway, in relation with Fyn, is associated with cancer progression in various cancer types, including melanoma. Research literature emphasizes the significance of natural compounds in cancer suppression and therapy ([19](#)).

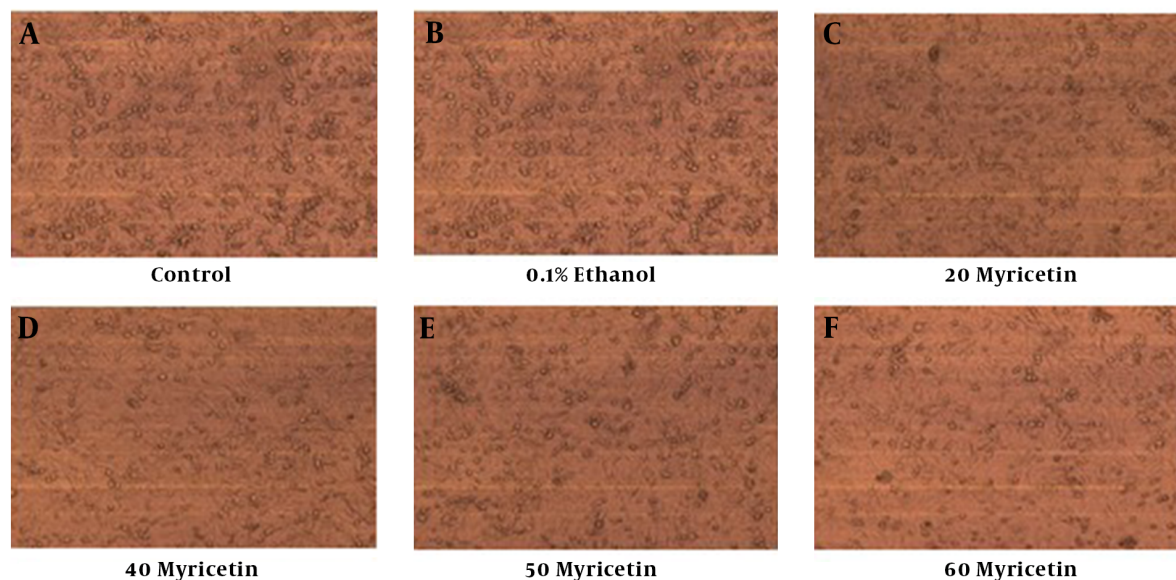
The Src has been identified as an important oncogene, yet little attention has been paid to its family members, including Fyn. Since Akt activation is common in many cancers, including prostate cancer, Fyn overexpression results in promotion of Akt anti-apoptotic activity ([13](#)). In breast cancer, Fyn expression is dependent on PI3K function. In this way, the Ras/ PI3K/ Akt pathway demonstrates overexpression of Fyn in cancer. These results support the advancement of therapeutic strategies targeting Fyn to suppress invasion and migration of tumor cells ([20](#)).

The current results determined that myricetin has an inhibitory effect on proliferation of melanoma cells (A375). The IC_{50} of myricetin for 24, 48, and 72 hours was 50, 40, and 35 μM respectively ([17](#)). In this study, the researchers concluded that exposure of cells with different concentrations of myricetin (0, 20, 40, 50, and 60 μM) after 48 hours, developed the expression of Fyn at mRNA level, dose dependently.

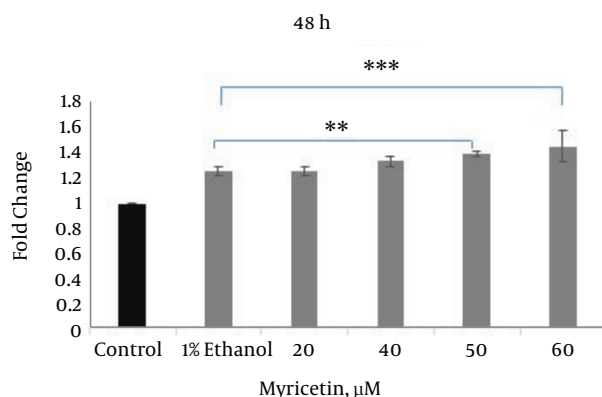
Pathologically, it has been demonstrated that low expression level of FRK protein could be involved in tumorigenesis and cell movement of human cervical cancer, so that FRK expression may be performed as a hopeful predictive indicator of this malignancy ([21](#)). In another research, it was shown that Fyn has a key role in signal transformation and proliferation of neuroblastoma cells. Therefore, the results detected that Fyn kinase expression suppresses tumor progression and metastasis ([22](#)). In agreement with these studies and based on the multifunctional role of Fyn in various cancer cells, it has been implied that in cancer cells like lung cancer, Fyn-related kinase led to cell growth, invasion, and colony formation of cells ([23](#)), while in some cancer cells, including colon, breast and hepatocyte cancer, the expression of Fyn kinase increased ([24](#)).

Overall, the achieved data on myricetin demonstrates its therapeutic effect of a multipurpose nature. The potential of myricetin in down-regulation of the oncogenic signaling pathways, such as PI3K, AKT/mTOR/p70SK and GSK-beta catenin pathways, could be a confirmed approach in treating human malignancies ([25](#)). Fyn, an oncoprotein, plays a critical role in the development of skin cancer under UVB-induced circumstances. Aberrantly, expression of Fyn in many human skin cancer cases, makes it an ideal target in treatment of skin cancer. Myricetin applies its chemo preventive effect against skin cancer by targeting the Fyn protein. To block UVB-mediated skin cancer, the molecular mechanism of myricetin involves the inhibition of Fyn protein, regulation of Cox-2 expression, and reduction in phosphorylation of MAPKs ([26](#)).

Ye Li et al. in 2016 detected that myricetin could reduce pro-inflammatory cytokines like IL-6, IL-1 β , and TNF- α to evaluate APC in mice. Also myricetin down regulates the phosphorylated P38 MAPK/ Akt/ m TOR signaling pathways

Figure 4. Treated Cells with Different Concentrations of Myricetin for 48 Hours

A, B, C, D, E, and F show treated cells with different concentrations of myricetin including: 0, 0.1% ethanol, 20, 40, 50, and 60 μM myricetin, respectively.

Figure 5. Calibrated Result of Real Time qPCR, Before and After Treatment with Different Concentrations of Myricetin

Each bar denotes the mean \pm SD of 6 experiments (** $P < 0.01$, *** $P < 0.001$ versus 0.1% ethanol group).

to inhibit adenomatous polyps (27). Myricetin with high cytotoxicity effect has chemo preventive impact on ovarian cancer cells like OVCAR-3 and A2780/CP70, which could prompt apoptosis through BCL-2 family proteins. In this regard, myricetin could induce P53, which plays an important role in BCL-2 family protein dependent apoptosis in cancer cells with no efficacy in promoting apoptosis in normal cells (28).

In some studies it has been shown that FRK functions as a tumor repressor. For instance, in breast cancer cells, the methylation of CPG sites in the promoter region of tumor suppressor was observed, and DNA methylation decreased the expression of FRK in a subset of breast cancer cells (29).

In this study, the researchers determined that myricetin could suppress the proliferation of melanoma cells, on the other hand, it did not have any toxicity effect up to 25 μM . Real time qPCR showed that myricetin could increase the expression of Fyn at mRNA level, so that increasing the concentration of myricetin, developed Fyn expression, as a tumor repressor, not noticeably.

Accumulated data have suggested that flavonoids, by directly interacting with protein kinases, could utilize their bioactivity to modulate intracellular signaling cascades and carcinogenesis. Different flavonoids, including myricetin, could bind to the ATP-binding site of Fyn (30). Since flavonoids could target multiple signaling pathways simultaneously, it is possible that cross-reactivity with unrelated ATP-binding proteins results in adverse consequences that cause many protein kinase inhibitors to fail (31).

On the other hand, in the signaling pathway, almost all components were identified, yet there is a possibility that they could activate, modify, or inhibit each other or may be involved in cross-reactivity to other pathways. For instance, GSK-3 β interacts with multiple signaling pathways,

such as PTEN/ PI3K/ AKt/ mTOR, Ras/ Raf, which could be regulated by tyrosine kinases, such as Src, Fyn, and Protein Tyrosine Kinase 2 beta (PYK2) (32), thus, these mutual reactions reveal that signaling pathways in malignant melanoma may compensate each other to make resistance to molecular-targeted therapy (33). Although it is hard to prognosticate the physiological or pharmacological importance of the inhibition potential of flavonoids, determining the expression of Fyn protein by western blotting could acknowledge our results on the basis of myricetin potential in decreasing the oncogenic effect of Fyn on malignant melanoma cells.

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Footnote

Conflicts of Interests: None declared.

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