Anti-leukemic effects of the quercetin on human leukemia U937 cells mediated by down-regulation of Mcl-1, survivin, and XIAP

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ABSTRACT
Recently, flavonoid quercetin is described as a natural product capable of the treatment of various types of leukemia, such as acute myeloid leukemia (AML), by targeting various survival and proliferation-associated signaling pathways or molecules. Herein, we evaluated the anti-leukemic effects of the quercetin on the human AML cell line, U937. Accordingly, the proliferation rate of the U937 cells was examined using MTT assay at 6, 12, and 24 hours of treatment with a series of quercetin concentrations, including 10, 20, 30, 40, 80, and 120 µM. Moreover, the apoptosis rates of U937 cells were estimated 6, 12, and 24 hours after exposure to quercetin 30 µM using annexin-V/PI staining and fluorescence-activated cell sorting (FACS) analysis. Finally, the expression rates of survivin, Mcl-1, XIAP, Bcl-2, and Bax were measured after treatment with quercetin 20 and 40 µM using Real-Time PCR within 6 and 12 hours of treatment. Concerning results, quercetin induced significant apoptosis in U937 cells within 6, 12, and 24 hours of treatment. Moreover, results verified the inhibitory effect of quercetin on U937 cell proliferation, more powerfully at 24 hours of exposure. Additionally, quercetin robustly modified Mcl-1, XIAP, and survivin expression at mRNA levels without any effect on Bax expression. Besides, this flavonoid stimulated slight but significant inhibitory effects on Bcl-2 expression at mRNA levels. In sum, the encouraging consequences of using quercetin toward the U937 cells have made it a favorable compound for treating AML through the downregulation of anti-apoptotic proteins.

Keywords: quercetin, acute myeloid leukemia (AML), proliferation, apoptosis, anti-apoptotic proteins

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INTRODUCTION

Acute myeloid leukemia (AML), as an aggressive type of leukemia, commonly targets the regulated differentiation and growth process of human hematopoietic myeloid lineage\textsuperscript{1,2}. Based on observations, a variety of chromosomal translocations and mutations in genes contributed to the hematopoietic growth, and differentiation can affect normal hematopoietic lineage cells and make them cancerous, thereby stimulating AML\textsuperscript{2}.

Currently, phytochemicals, in particular, flavonoids have concerned great attention among researchers around the world with the aim of leukemia treatment\textsuperscript{3-6}. Flavonoids are extensive types of phytochemicals commonly detected in fruits, vegetables, tea, soy, wine, and also medicinal plants\textsuperscript{7}. They typically elicit inhibitory effects on human leukemia cell's proliferation and survival because of their competence to modify vital signaling molecules that participated in cell proliferation and apoptosis\textsuperscript{8-11}. It has been suggested that quercetin, as a significant flavonoid, could be used as a potential therapeutic ingredient because could inhibit a myriad of leukemia-associated processes containing oxidative stress, apoptosis, proliferation, and metastasis\textsuperscript{12}. For example, an investigation of quercetin effect on human leukemia HL-60 revealed that it resulted in leukemia cells apoptosis by promoting the pro-apoptotic protein Bax levels, along with inhibition of the Bcl-2 protein levels, and induction of caspase-2 and -3, and increased poly (ADP-ribose) polymerase cleavage\textsuperscript{13}. Likewise, quercetin caused a noticeable suppression of human leukemia K562 cell proliferation concurrently mild cytotoxicity, as shown by stimulated caspase-3 and cytochrome C-dependent apoptosis and an improvement of cell frequencies in the G2/M phase\textsuperscript{14}. As well, combination therapy with quercetin and high-dose adriamycin could prominently promote the overall survival rate of non-irradiated leukemia mice and decrease the myocardial lesions stimulated by adriamycin\textsuperscript{15}. 
In the present study, we examined the possible anti-leukemic effects of the quercetin on human leukemia U937 cell proliferation using Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay, and also apoptosis by fluorescence-activated cell sorting (FACS) analysis. Moreover, we estimated the expression level of the survivin, Bcl-2, Mcl-1, Bax, and XIAP contributed to cell proliferation and survival by Real-Time PCR (RT-PCR) to address whether quercetin has any capability to modify their expressions in U937 cells.

MATERIALS and METHODS

Cell culture

The human AML cell line U937 was acquired from Iran Pasteur Institute (Tehran, Iran), and cultured in RPMI 1640 medium (Sigma-Aldrich/Merck KGaA Darmstadt, Germany) with 10% fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific, Waltham, MA, US) and maintained at 37°C with 5% CO².

Reagents

The stock solution of quercetin was primed by quercetin powder (quercetin ≥95.0% purity, Sigma-Aldrich, Germany). Also, DMSO and MTT reagents were prepared from Sigma-Aldrich Germany.

MTT assay

The cytotoxicity of quercetin in the U937 cells was examined using the MTT assay concerning MTT kit instructions. Concisely, 1×10⁴ U937 cells/100 µL of RPMI-1640 medium were seeded into the wells of a 96-well plate, and after that quercetin was added at 10-120 µM concentrations into the U937-containing wells. Upon three time-points; 6, 12, and 24 hours of exposure, 20 µL of 5 mg MTT/ml medium was added into U937-containing wells. Cells were maintained at 37°C for 4 hours, and then the optical density (OD) of wells was estimated at 570 nm wavelengths with an ELISA reader.

Flow cytometric analysis of apoptosis

An annexin V-based kit (Apoptotest™-FITC Kit, Dako, Glostrup, Denmark) was utilized to estimate the apoptosis levels of the U937 cells after treatment with quercetin 30 µM within 6, 12, and 24 hours of exposure. After adding the 5 µL of propidium iodide (PI) and 5 µL of fluorescein isothiocyanate (FITC)-conjugated annexin-V into U937-containing wells, the apoptosis percentages were measured rendering fluorescent signal emission from the FITC-annexin bound phosphatidylserines. The fluorescent emission was sensed by a FACSCalibur machine (BD Biosciences, Franklin Lakes, NJ, US) and the consequences were analyzed by FlowJo software (v 10.4.1).

Real-Time quantitative PCR (RT-qPCR)

The primer-blast software on the NCBI website was utilized to assay primer sequences described in Table 1 for their specific binding. Moreover, RNA isolation was carried out utilizing Trizol reagent (Invitrogen, Milan, Italy) and complementary DNA (cDNA) was manufactured by the PrimeScript™ reagent Kit (Takara Bio, Kusatsu, Japan). Real-time PCR tests were executed in triplicates using the RealQ Plus 2x Master Mix Green (Ampliqon, Herlev, Denmark). Also, the GAPDH gene was used as the internal control.

Statistical Analysis

Statistical analyses were carried out by GraphPad Prism version 8.01. The consequences were illustrative of means ± SEM of the triplicate test. The student’s t-test was applied to determine statistical differences among the investigational groups. P-value <0.05 was considered statistically significant.

RESULTS

Quercetin mitigated U937 cells proliferation

Concerning MTT assay consequences, quercetin 10,

| Gene   | Primer (5’-3’) | Primer (5’-3’) |
|--------|---------------|---------------|
| Survivin | F TCTCAAGGACCACCCGATC | R GCCAGTCTGGCTCTTC |
| Bcl-2  | F TGCCCTGTTAGTGACTGAG | R CAGACTTTCAGAAGCCAGGA |
| XIAP   | F ATATGTCAGCAGCTACCA | R CAGATGGGCTTCTTGAAGGCA |
| Mcl-1  | F AGAAGGCTGATCGAACCAT | R CCAGCTCTACTCCAGCAAC |
| Bax    | F TTTGGCTCAGGGTTCATCC | R GCCATCGAGAAAGACCT C |
| GAPDH  | F TGATGACATCAAGAGTGTTGAAG | R TCTTGGAGGCCATGTTGGCCAT |
Quercetin anti-leukemic effects

20, 30, 40, 80 and 120 µM inhibited U937 cell proliferation, slightly or strongly (P < 0.05) (Figs. 1A, 1B). Quercetin 10 µM did not affect the proliferation of the U937 cells within 6 hours of treatment, while significantly modified U937 cells proliferation 12 and 24 hours of exposure (P < 0.05) (Figs. 1A, 1B). Also, quercetin at 20, 30, 40, 80, and 120 µM concentrations remarkably diminished U937 cell proliferation within 6, 12, and 24 hours of exposure (P < 0.05) (Figs. 1A, 1B). According to results, the inhibitory effects of the quercetin on U937 cell proliferation were more prominent at 120 µM concentration and 24 hours of exposure than other concentrations and incubation periods (P < 0.05) (Figs. 1A, 1B).

**Quercetin induced U937 cells apoptosis**

Results revealed a significant shift in apoptosis percentages in U937 cells-incubated with quercetin 30 µM compared with control cells (untreated U937 cells) during 6, 12, and 24 hours of treatment estimated by annexin-V/PI staining and FACS analysis (P < 0.05) (Figs. 2A, 2B).
Based on consequences, apoptosis percentage during 24 hours of treatment was higher than 6 and 12 hours of treatment ($P > 0.05$) (Figs. 2A, B). Accordingly, apoptosis percentages in U937 cells (control), those treated with quercetin 30 µM within 6 hours of exposure, those treated with quercetin 30 µM within 12 hours of exposure and those treated with quercetin 30 µM within 24 hours of exposure were $4.18\pm1.43$, $19.21\pm2.65$, $42.04\pm3.01$, and $59.08\pm2.41\%$ of total cells, respectively (Figs. 2A, 2B).

**Quercetin reduced Mcl-1 and XIAP expression in U937 cells**

According to Real-Time PCR results, Mcl-1 and XIAP expression rates were diminished in U937 cells after treatment with quercetin 20 and 40 µM at 6 and 12 hours of treatment ($P < 0.05$) (Figs. 3A, 3B).
both 6 and 12 hours of treatment, the inhibitory effect of quercetin on Mcl-1 and XIAP expression was higher in quercetin at 40 µM than quercetin at 20 µM (P < 0.05) (Figs. 3A, 3B). Though differences in expression rates of XIAP between groups treated with quercetin 20 µM and 40 µM were not statistically significant within 6 hours of exposure, there were significant differences between treated groups at 12 hours of exposure (P < 0.05) (Fig. 3B). Also, there was a significant difference between groups treated with quercetin 20 µM and 40 µM within 6 and 12 hours of exposure in terms of Mcl-1 expression (P < 0.05) (Fig. 3A).

**Quercetin reduced survivin expression in U937 cells**

The survivin gene expression did not significantly modify in U937 cells within 6 hours of treatment with quercetin 20 µM, while analysis showed a significant attenuation in survivin expression after treatment with quercetin 20 µM at 12 hours of exposure (P < 0.05) (Fig. 3C). Moreover, quercetin 40 µM attenuated survivin expression within 6 and 12 hours of incubation (P < 0.05) (Fig. 3C). But, there was no significant difference between survivin expression in U937 cells treated with quercetin 20 µM and 40 µM during both 6 and 12 hours of exposure (P < 0.05) (Fig. 3C).

**Quercetin slightly diminished Bcl-2 expression in U937 cells**

Based on consequences, although quercetin 20 µM attenuated Bcl-2 expression in U937 cells within 6 and 12 hours of treatment, this reduction was not statistically significant (Fig. 4A). Moreover, quercetin 40 µM did not affect Bcl-2 expression in U937 cells within 6 hours of treatment, while the analysis showed a significant reduction in Bcl-2 expression at 12 hours of treatment with quercetin 40 µM (P < 0.05) (Fig. 4A). On the other hand, there was no significant difference between Bcl-2 expression in U937 cells treated with quercetin 20 µM and 40 µM during both 6 and 12 hours of exposure (Fig. 4A).

**Quercetin did not modify Bax expression in U937 cells**

Based on the consequence, the expression level of Bax did not change in U937 cells after exposure with quercetin 20 µM and 40 µM during both 6 and 12 hours of exposure.
exposure (Fig. 4B). According to Real-Time PCR results, although quercetin 20 µM and 40 µM promoted Bax genes expression levels at 6 and 12 hours of exposure, the improvement was not significant (Fig. 4B).

**DISCUSSION**

The treatment option toward AML commonly is consists of the two-phase therapy program, encompassing induction therapy and post-remission (consolidation) therapy30. As well, anti-cancer chemotherapy, which is considered a conventional therapeutic strategy for AML therapy, is poisonous to both normal hematopoietic cells and AML cells, and thereby normal hematopoietic cells are also eradicated through anti-cancer chemotherapy37. In this regard, several unwanted events including anemia, thrombocytopenia, neutropenia, and monocytopenia are shown after chemotherapy in patients suffering from leukemia18-20. On the other hand, the incidence of serious untoward effects such as graft versus host disease (GVHD) hinders the safety and efficacy of stem cell transplantation in AML patients21, 22. Thereby, researchers try to find innovative and more effective therapeutic agents or approaches to attenuate negative effects.

Currently, flavonoids because of their unique attributes particularly lower side effects and higher safety are introduced as promising candidates against leukemia23, 24. Flavonoids can robustly target crucial biologic procedures of the malignant cells and thereby obstruct their growth and viability through affecting important gene expression and protein functions25-27. Quercetin, a well-known flavonoid, could attenuate the growth of the human HL-60 leukemia cell line and also induced cell cycle arrest in treated cells by inhibition of protein kinase C (PKC) and tyrosine-protein kinase (TKP) functions28. Moreover, in vivo studies in leukemic NOD/SCID mice models have suggested that quercetin could reduce the expression of anti-apoptotic proteins, Bcl-2, Bcl-xL, and Mcl-1, and also promote Bax expression. As well, this flavonoid stimulated caspase-3 activation resulted in leukemic cell apoptosis in treated models29.

Furthermore, Naimi et al. found that quercetin could stimulate human leukemia KG-1 cells apoptosis and inhibit their proliferation by targeting anti-apoptotic protein c-FLIP and XIAP. Also, they showed that quercetin enhanced KG-1 cell sensitivity to TNF-related apoptosis-inducing ligand (TRAIL)30. Other investigations have demonstrated that Bax, caspase-3, and caspase-8 expression was considerably amended in human leukemia K-562 cells after exposure with quercetin, which in turn, led to the apoptosis induction in K-562 cells31. Based on findings it seems that down-regulation of anti-apoptotic proteins plays a pivotal role in quercetin-elicited anti-leukemic effect in vitro and in vivo. Meanwhile, it has been verified that quercetin significantly promoted the down-regulation of Mcl-1 in B cells procured from selected patients showing measurable rates of Mcl-131.

In the present study, we aimed to evaluate the anti-proliferative and apoptosis-inducing capacity of quercetin in human leukemia U937 cells. Accordingly, we estimated the gene expression levels of the survivin, Bcl-2, Mcl-1, XIAP, and Bax in quercetin-exposed U937 cells to clarify the likely anti-leukemic mechanism of action of quercetin. We found that quercetin could abrogate the U937 cell proliferation in a time-dependent and dose-dependent manner. As well, this flavonoid induced U937 cells apoptosis during 6–24 hours of exposure. On the other hand, the intense suppressive impacts of quercetin toward anti-apoptotic Mcl-1, XIAP and survivin expression levels showed that quercetin possibly stimulated apoptosis in treated cells by inhibition of these protein expressions, supporting the activation of caspases-3, -8, -9. However, we surprisingly found that quercetin could slightly modify Bcl-2 expression only 24 hours after treatment. More surprisingly, results showed that quercetin could not affect Bax expression in U937 cells at mRNA levels. We guess that quercetin could stimulate Mcl-1 down-regulation, thereby facilitating Bax activation and mitochondrial translocation, leading to apoptosis induction in U937 cells. As well, inhibition of survivin and XIAP expression resulted in activation of caspases cascades was found could be another underlying mechanism in this course.

In sum, we found that quercetin could abrogate the proliferation of U937 cells and promote these cell apoptosis by down-regulation of Mcl-1, survivin, and XIAP and also induction of Bax activation, which in turn, supported caspase cascade activation. As well, we suggest that quercetin can be a favorite ingredient to develop an effective anti-leukemic therapeutics strategy against leukemia with minimized side-effects and a suitable rate of safety. However, further consideration and more studies are required to explain the meticulous pharmacology and toxicology of flavonoids once applied as a therapeutic compound against leukemia.

Conflict of Interest:
The authors declare that there are no conflicts of interest.

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