Flavonoids kaempferol (KAE) and quercetine (QUE) inhibited proliferation of human leukemia THP-1 cells by up regulation of pro-apoptotic protein Bax and caspase 3/8 expression and down regulation of anti-apoptotic proteins Bcl-2, Bcl-xl and Mcl-1 expression

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Abstract
Acute myeloid leukemia (AML) is a type of leukemia robustly affecting the normal proliferation and maturation procedure of human hematopoietic myeloid lineage. Nowadays, Flavonoids, well-known types of natural product, because of their acceptable efficacy and lower side effects have attracted increasing consideration in the context of AML therapy using natural products and herbal medicine. Herein, we evaluated flavonoid kaempferol (KAE) and quercetin (QUE) on acute myeloid leukemia THP-1 cells proliferation. To address the anti-leukemic potential of KAE and QUE in leukemia THP-1 cells, these cells were treated with KAE (40 µM), QUE (40 µM), and KAE plus QUE within 12, 24, 48, and 72 hours of exposure. Then, cell proliferation was evaluated using methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Moreover, expression rates of the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2, Mcl-1, and Bcl-xl along with caspase 3 and caspase 8 were assessed by real-time PCR (RT-PCR) during 24 and 48 hours of exposure with KAE (40 µM), QUE (40 µM), and KAE plus QUE. After that, the candidate’s gene expression levels were compared with control THP-1 cells. Based on MTT assay results, KAE and QUE at 40 µM concentration reserved proliferation of THP-1 cells compared with control cells, while the anti-proliferative effects of the QUE had superiority over KAE in treated cells. Importantly, results evidenced the synergistic effects of the KAE plus QUE on THP-1 cell proliferation during all periods of the experiment. On the other, these compounds could improve Bax, caspase 3, and caspase 8 expressions, and conversely stimulated a significant and robust reduction in Bcl-2, Mcl-1, and Bcl-xl expression at mRNA levels in the treated cell compared with control cells. In sum, we suggested that the use of the KAE plus QUE could more evidently abrogate proliferation and viability of human leukemia THP-1 cells targeting survival involved genes expression, delivering the proof of the concept that combines the application of KAE and QUE is a rational strategy to treat AML.

Keywords: Acute myeloid leukemia (AML), Flavonoids, THP-1 cells, Proliferation

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INTRODUCTION
Acute myeloid leukemia (AML) is a type of leukemia sturdily targeting the normal proliferation and maturation process of human hematopoietic myeloid lineage1-2). A complex of unwanted proceedings containing the chromosomal translocations and mutations in genes, which control the hematopoietic proliferation and maturation, resulted in AML onset3-4). There exist various undesirable attributes of AML leading to the poor prognosis and disappointing therapeutic outcome, in particular, higher risk of recurrence along with resistance to anti-cancer chemotherapies and other conventional approaches5). Nowadays, researchers have believed that the discovery and analysis of drug candidates showing acceptable efficacy in concomitant with no/minimum untoward effect and toxicity toward normal hematopoietic cells are of paramount importance6). Accordingly, phytochemicals (e.g., flavonoids) have attracted great attention among researchers around the world to treat AML.

Flavonoids are extensive types of phytochemicals found in fruits, vegetables, tea, soy, wine, and medicinal plants7-10). Importantly, flavonoids not only can
hinder cancer progress but also their metabolites are capable of induction of malignant cells susceptibility to conventional chemotherapies\textsuperscript{11,12}. Based on studies, the existence of a correlation between flavonoids and a wide-ranging of health-improving functions described them as an essential part of many experimental herbal therapies and medicinal uses. Flavonoids perform as inhibitory ingredients toward human leukemia cells because of their capabilities to modulate pivotal signaling molecules that contributed to cell proliferation and apoptosis\textsuperscript{13-18}. Kaempferol (KAE) and quercetin (QUE) are well-known flavonoids existing in plants and plant-derived foods with a wide variety of pharmacological possessions such as anti-leukemic properties. It has already been shown that KAE averts proliferation and attenuates the viability of human leukemia K562 and U937 cells upon suppression of PI3K/Akt signaling pathways\textsuperscript{17}. As well, it triggered acute promyelocytic leukemia (APL) cell apoptosis, as evidenced by amelioration in the sub-G1 population, possibly mediated by inhibition of Bcl-2 gene expression\textsuperscript{18}. Other reports indicated that QUE suppressed human leukemia HL-60 cell proliferation and induced apoptosis upon inhibition of Bcl-2 expression and augmentation of pro-apoptosis protein Bax expression\textsuperscript{19}. On the other hand, there is some evidence supporting that flavonoid QUE inhibited human leukemia KG-1 cell proliferation and improved TRAIL-mediated apoptosis in these cells via up-regulation of death receptors 4/5 (DR4/5) expression and downregulation of anti-apoptotic protein c-FLIP, X-IAP, and survivin\textsuperscript{19}. In the present study, we reported the cytotoxicity of KAE and QUE against human monocytic cell line THP-1 using methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay upon exposure with KAE and UQE, alone and together. Next, the expression levels of the Bcl-2, Mcl-1, Bax, Bcl-xL, and caspase 3 and caspase 8 genes were evaluated by real-time PCR (RT-PCR) at mRNA levels.

MATERIALS AND METHODS

Cell culture

The human monocytic cell line THP-1 was acquired from Pasteur Institute of Iran (Tehran, Iran), and cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, Thermo Fisher Scientific, US) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, US) and then maintained at 37°C with 5% CO\textsubscript{2}.

Reagents

A stock solution of KAE and QUE was primed by KAE and QUE powder (Sigma-Aldrich, Germany). Moreover, dimethyl sulfoxide (DMSO) and MTT reagents were purchased from Sigma-Aldrich (Sigma-Aldrich, Germany).

MTT assay

The examination of the cytotoxicity of KAE and QUE against THP-1 cells was carried out by the MTT assay based on manufacturer instructions. Concisely, 1×10\textsuperscript{4} THP-1 cells/100 µL of RPMI-1640 medium were seeded into the wells of a 96-well plate, and then the KAE and QUE at 40 µM concentrations were added into THP-1-containing wells alone and combined with. At four time-points; 12, 24, 48, and 72 hours after cell exposure with flavonoids, 20 µL of 5 mg MTT/ml medium was added into the wells. After that, cells were incubated at 37°C for 4 hours, and the optical density (OD) of wells was estimated at 570 nm wavelength with an ELISA reader.

Real-Time quantitative PCR (RT-qPCR)

The primer-blast software on the NCBI website (http://www.ncbi.nlm.nih.gov) was employed to evaluate primer sequences, which are listed in Table 1. The RNA isolation was executed by Trizol reagent (Invitrogen, Milan, Italy) and complementary DNA (cDNA) was manufactured applying the PrimerScript\textsuperscript{™} reagent Kit (Takara, Japan). Real-time PCR assays were conducted in triplicates by the RealQ Plus 2x Master Mix Green (Ampliqon, Herlev, Denmark). GAPDH gene was used as the internal control.

Statistical Analysis

Statistical analyses were accomplished by GraphPad Prism version 8.01. The MTT and RT-PCR assay sequences were descriptive of means ± SEM of three independent experiments. One-way ANOVA was done to determine statistical differences among the experimental groups. Also, P-values <0.05 were considered statistically significant.

RESULTS

| Gene | Primer (5′ -3′) |
|------|----------------|
| Bcl-2 | F TCGCCCTGTTGATGACTGAG |
| R CAGAGTCTTCAGAGACGCCAGGA |
| Bcl-xL | F GTTCCTTCTCTCATCC |
| R TAGCCAGTCCAGAGGTTGAG |
| Mcl-1 | F AGAAAAGCTGACATCGAACCAT |
| R CCAAGCTTCACTCCAGCAAC |
| Caspase 3 | F TGAGCCATGTGGAAGAGAAGA |
| R TCGGCCCTCCTGCTGGATATTT |
| Caspase 8 | F CAGCAGGGGCTAAAATTTCT |
| R TCTGCCACCTTCTGCTGAAATCTGA |
| Bax | F TTGTGCTTCAGGGTTCATCC |
| R GCCACCTCGGAAAAAAGACCTC |
| GAPDH | F TGATGACATCAGAGAAGTGGTGAAG |
| R TCTTTGAGGCCATGTTGCGC |

KA3 and QUE inhibited THP-1 cells proliferation

THP-1 cells were treated with KAE (40 µM), QUE (40 µM), and DMSO for 4 hours. The sub-G1 population was evidenced by amelioration in the sub-G1 population, positively correlated with the RealQ Plus 2x Master Mix Green (Ampliqon, Herlev, Denmark). GAPDH gene was used as the internal control.

Table 1. Candidate genes’ primer pairs for Real-Time PCR

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µM), and KAE plus QUE within 12, 24, 48, and 72 hours of exposure. According to MTT assay results, KAE and QUE at 40 µM concentration abrogated proliferation of THP-1 cells at all experimental periods (P < 0.05) (Figs. 1A, B). However, this reduction was more obvious 72 hours of incubation than 12, 24, and 48 hours of exposure (P < 0.05) (Figs. 1A, B). Moreover, the inhibitory effect of the QUE 40 µM on THP-1 cell proliferation had superiority over KAE anti-proliferative activity within 12, 24, 48, and 72 hours of incubation (P < 0.05) (Figs. 1A, B). Importantly, results demonstrated that combined use of the KAE and QUE could avert THP-1 cell proliferation more evidently than groups treated with KAE and QUE alone, during all periods of the experiment (P < 0.05) (Figs. 1A, B).

KAE and QUE significantly promoted Bax/Bcl-2 expression ratio in THP-1 cells

The RT-PCR analysis showed that QUE 40 µM could improve Bax expression at mRNA levels alone and combined with KAE 40 µM within 24 hours of exposure, while KAE 40 µM could promote Bax expression slightly but not significantly in treated THP-1 cells compared with the control group during same periods (P < 0.05) (Fig. 2A). During 48 hours of treatment, all treated groups showed statistically significant amelioration in terms of Bax expression in comparison with control THP-1 cells (P < 0.05) (Fig. 2A). The increase in Bax expression levels was more evident in groups treated with KAE plus QUE compared with groups treated with KAE and QUE alone (P < 0.05) (Fig. 2A).

On the other hand, Bcl-2 expression was abridged upon THP-1 cell treatment with KAE and QUE 40 µM alone and together with 24 and 48 hours after exposure compared with the control group (P < 0.05) (Fig. 2B). The inhibitory effects of the QUE 40 µM in Bcl-2 expression at mRNA levels were more prominent than KAE 40 µM inhibitory effects. Furthermore, the analy-
sis showed a statistically significant difference in Bcl-2 expression between groups treated with KAE and QUE alone compared with groups treated with KAE plus QUE within 24 and 48 hours of treatment (P < 0.05) (Fig. 2B).

**KAE and QUE significantly down-regulated Bcl-xL and Mcl-1 expression in THP-1 cells**

Analysis evidenced that Bcl-xL expression rate was reduced upon THP-1 cell exposure with KAE and QUE 40 µM alone and combined during 24 and 48 hours of incubation compared with the control group (P < 0.05) (Fig. 3A). Moreover, results revealed a significant decrease in Bcl-xL expression at mRNA levels between groups treated with KAE and QUE alone compared with groups treated with KAE plus QUE within 24 and 48 hours of exposure (P < 0.05) (Fig. 3A). Besides, While Mcl-1 expression was attenuated upon THP-1 cells treatment with KAE and QUE 40 µM alone and combined during 24 of incubation compared with the control group, this reduction in groups treated with KAE was not significant (P < 0.05) (Fig. 3B). However, KAE 40 µM could stimulate a significant reduction in Mcl-1 expression levels within 48 hours of treatment (P < 0.05) (Fig. 3B). On the other hand, diminishment in Mcl-1 expression levels was not statistically significant between groups treated with QUE alone and groups treated with QUE plus KAE (P < 0.05) (Fig. 3B).

**KAE and QUE significantly up-regulated caspase 3 and caspase 8 expression in THP-1 cells**

According to consequences, caspase 3 and 8 gene expression levels were promoted within 24 and 48 hours of treatment in THP-1 cells treated with KAE and QUE 40 µM alone and in combined (P < 0.05) (Figs. 4A, B). Moreover, results presented a significant promotion in caspase 3 and 8 gene expression between groups treated...
with KAE and QUE 40 µM alone compared with cells treated with KAE plus QUE within 24 and 48 hours of incubation (P < 0.05) (Figs. 4A, B). Besides, there were significant differences between expression levels of caspase 8 but not caspase 3 between groups treated with KAE 40 µM and groups treated with QUE 40 µM alone during 24 and 48 hours of exposure (P < 0.05) (Fig. 4B).

**DISCUSSION**

Some unwanted features of AML resulted in the poor prognosis and undesired therapeutic outcome, including greater recurrence incidence rates concomitant with resistance to anti-cancer agents\textsuperscript{20-21}. Today, researchers effort to design or find novel and innovative drugs or therapeutic approaches to elicit suitable efficacy in the absence of any severe unwanted effect to treat AML\textsuperscript{22}. Accordingly, natural products such as flavonoids have concerned pronounced consideration among scholars worldwide for AML therapy\textsuperscript{13, 23}. QUE and KAE, important polyphenols found in a wide spectrum of plants, have been described that pose cytotoxic effects on various types of cancer (e.g. leukemia)\textsuperscript{16, 24}.

It has already been found that QUE and KAE could stimulate apoptosis in human erythroleukemic K562/ A by down-regulation of anti-apoptotic protein Bcl-2\textsuperscript{25}. Other reports supported that QUE abridged tumor growth in human myeloid leukemia HL-60 xenografts in concomitant with attenuated expression of anti-apoptotic proteins, Bcl-2, Bcl-xL, and Mcl-1, and augmented expression of Bax, a pro-apoptotic protein\textsuperscript{26}. On the other hand, Jacquemin et al. found that QUE restored TRAIL-induced apoptosis in resistant transformed leukemia cells mediated by the promotion of the proteasomal degradation of Mcl-1 and by suppression of the survivin expression at the mRNA level\textsuperscript{27}. Another flavonoid, KAE, could trigger apoptosis and abrogate proliferation of AML cells upon promotion of Bax/Bcl-2 expression ratio and caspase 3 expressions, as evidenced by Western blot analysis. The consequences suggested KAE reduced cell viability and improved subG1 population in treated cells\textsuperscript{30}.

There exist consistency between these results and the consequences of the present study. Herein, we showed that KAE and QUE inhibited human monocytic leukemia THP-1 cell proliferation when used alone or combined with. However, the inhibitory effect of the QUE was more prominent than KAE at the same concentration (40 µM). Also, results demonstrated a time-dependent manner and synergistic effect of the two flavonoids on THP-1 cells within 12, 24, 48, and 72 hours of exposure (Fig. 1). Analysis of the expression levels of candidate genes at mRNA levels by RT-PCR clarified the corresponding mechanism of the inhibitory effect of the KAE and QUE on experimental cell viability. Results revealed that both KAE and QUE could attenuate Bcl-2, Bcl-xL and Mcl-1 expression resulted in a reduction in cell viability (Fig. 2 and 3). Besides, these compounds up-regulated pro-apoptotic protein Bax expression, leading to the promotion of Bax/Bcl-2 expression ratio in THP-1 cells upon treatment. Comparative analysis verified that combined use of the KAE and QUE had superiority in terms of the downregulation of anti-apoptotic protein and up-regulation Bax over these compound use, lonely. Moreover, KAE and QUE elicited substantial upregulation in caspase 3 and caspase 8 expressions in THP-1 cells (Fig. 4). Accordingly, results evidenced the synergistic effect of the two flavonoids on candidate gene expression at mRNA levels in THP-1 cells. Similarly, other studies demonstrated that KAE triggered morphological variances and internucleosomal DNA fragmentation characteristic of apoptosis possibly exerted by caspase 8 and caspase 3 activation and cytochrome c release in leukemia HL-60 cells\textsuperscript{28}. Moreover, Gokbulut and his colleagues found that QUE obstructed proliferation of human 232B4 chronic lymphocytic leukemia cells by caspase 3 upregulation and induction of cell cycle arrest\textsuperscript{29}.

In sum, consequences showed the synergistic impacts of the KAE plus QUE on THP-1 cell proliferation. Molecular analysis revealed that both KAE and QUE can up-regulate Bax, caspase 3, and caspase 8 expressions, and conversely, down-regulated Bcl-2, Mcl-1, and Bcl-xL expression treated cells. Accordingly, it seems that the promotion of the Bax/Bcl-2 expression ratio plays a pivotal role in the anti-leukemic effects elicited by these flavonoids in THP-1 cells. Although the multifactorial activities of KAE and QUE and the molecular axis contributed to their functions in leukemia cells are poorly unstated and there are still few reliable clinical trials aiming to clarify the clinical applicability of these compound, we propose that combined use of the KAE and QUE can be authentically and effective strategy against a diversity of leukemia in concomitant with minimum side-effects and proper level of safety.

**Conflict of interest:**
There is no conflict of interest.

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