



Immunomodulatory effects of apigenin, luteolin, and quercetin through natural killer cell cytokine secretion

Aung Myo Oo¹, Mohd Nasir Mat Nor¹, Ohn Mar Lwin², Nordin Simbak³, Liyana Hazwani Mohd Adnan¹, Uthakar S Mahadeva Rao^{1*}

¹Faculty of Medicine, University Sultan Zainal Abidin, Kuala Terengganu, Malaysia.

²Faculty of Medicine, International Medical School, Management and Science University, Shah Alam, Malaysia.

³Faculty of Medicine, Bukhara State Medical Institute, Bukhara, Uzbekistan.

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ABSTRACT

Nutritional immunology has attracted the interest of researchers due to its positive impact on health and immunity. By secreting cytotoxic granules and producing cytokines, natural killer (NK) cells are pivotal in fighting against viruses and cancer. Polyphenolic secondary metabolites, like flavonoids, have potent immunomodulatory effects by influencing cytokines. This study aimed to demonstrate the cytokine production of flavonoid-stimulated NK cells to find alternative immunomodulatory effects on immune cells. NK-92 cells were supplemented with three flavonoid compounds, apigenin, luteolin, and quercetin, overnight. An enzyme-linked immunosorbent assay was used to measure the levels of type-1 cytokines, interleukin-2 (IL-2), interferon-gamma, and type-2 cytokines, IL-4, and IL-10. Luteolin and quercetin treatment significantly increased NK cells' IL-2 secretion but not apigenin. NK cell IFN- γ secretion was also significantly enhanced by luteolin at 25 μ g/ml concentration. Nonetheless, at different doses, NK cell IL-4 and IL-10 secretions were unaffected by any of the three flavonoid compounds tested. Luteolin has the greatest effect on NK cell type-1 cytokine production, whereas quercetin only produces IL-2. Meanwhile, apigenin had no effect on type-1 and type-2 cytokine secretion. It is postulated that luteolin and quercetin modulate immune function through type-1 cytokines while having no effect on type-2 cytokine production.

INTRODUCTION

Natural killer (NK) cells directly contribute to the immune-defence mechanism by performing effector functions such as cell-mediated cytotoxicity and protein cytokine secretion. The third commonest contributor of innate immunity is provided by NK cells after B and T lymphocytes, protecting the immune system's defences against viral infection and neoplastic cells (Hou *et al.*, 2017). In addition to direct target cell lysis by perforin and granzyme and induction of apoptosis, for activation of systemic immune-

mediated antitumor response, NK cells are able to produce numerous cytokines, for example, interferons, interleukins, tumor necrosis factor-alpha (TNF- α), and other proinflammatory cytokines and chemokines (Fauriat *et al.*, 2010; Paul and Lal, 2017). Furthermore, various bioactive compounds can augment the activity of NK cells, causing them to release a variety of cytokines, for example, IFN- γ , granulocyte-macrophage-colony-stimulating factor, and TNF- α (Deniz *et al.*, 2013; Vivier *et al.*, 2011). According to previous studies, type-1 helper T (Th1) cytokines such as interleukin-2 (IL-2) and interferon- γ (IFN- γ) promote cellular immunity (Wu *et al.*, 2017), while type-2 helper T (Th2) cytokines, namely, IL-4, IL-6, and IL-10, suppress cellular immunity while increasing antibody-mediated humoral immunity (Ferreira *et al.*, 2018). Although peripheral NK cells principally release type-1 cytokines, their ability to secrete type-2 cytokines has been demonstrated (Veenstra

*Corresponding Author

U. S. Mahadeva Rao, Faculty of Medicine, University Sultan Zainal Abidin, Kuala Terengganu, Malaysia. E-mail: raousm@gmail.com

van Nieuwenhoven *et al.*, 2002). The cytokines secreted by NK cells govern both innate and adaptive immunity (Degli-Esposti and Smyth, 2005). Cytokine expression can, however, be triggered by lipopolysaccharide (LPS), reactive oxygen species, and microbial species, among other things (Leyva-López *et al.*, 2016).

Nowadays, the interest in nutritional immunology has increased tremendously and established massive molecular connections between plant-derived bioactive compounds and their effects on immune cells. Many dietary secondary metabolites, such as flavonoids, show immune-stimulating properties on NK cell activity (Leischner *et al.*, 2015). There were studies of flavonol quercetin having a direct effect on cytokine gene expression and production of IL-4 and IFN- γ (Hosseinzade *et al.*, 2019; Nair *et al.*, 2002). Lutein, fisetin, and apigenin were the most effective basophil IL-4 and IL-13 production inhibitors. Furthermore, these flavonoids inhibited the secretion of IL-4 from T lymphocytes (Hirano *et al.*, 2004; Liang *et al.*, 2020). Low doses of flavonoid resveratrol, in contrast, were shown to induce immune responses in mice by increasing the production of IFN- γ , while its high dose promotes apoptosis of target cells (Lee *et al.*, 2020). Among flavonoid compounds, the two flavones, apigenin and luteolin, and flavonol quercetin were paid much attention as these natural compounds showed immunomodulatory and anticancer agents based on previous studies (Panche *et al.*, 2016). Previous research has shown that the flavones apigenin, luteolin, and flavonol quercetin promote NK-92 cell proliferation in a dose-dependent manner (Aung *et al.*, 2021). Similarly, we reported that increasing the concentrations of luteolin, apigenin, and quercetin dramatically improved the NK cells killing activity towards lung cancer by raising perforin and granulysin secretion (Oo *et al.*, 2020). Continuing from the previous study, we aimed to demonstrate that apigenin, luteolin, and quercetin display immunomodulatory activity by regulating NK cells' cytokine production.

MATERIALS AND METHODS

The *in vitro*, cell-line-based experiment was completed at the Cell Culture Laboratory, Medical Faculty, Universiti Sultan Zainal Abidin, Malaysia, in the year 2020. The laboratory

experiments were performed in a biosafety cabinet under strict sterile conditions (ESC II series, Erla Technologies).

Cell line

The American Type Culture Collection (ATCC) provided the natural killer-92 (NK-92) cell line (Elabscience, ATCC, USA). The NK cells were preserved in cryotubes and kept in liquid nitrogen prior to being thawed and cultured. NK cells were cultured in the complete α - Minimum Essential Medium (α -MEM) (Nacalai Tesque Inc., USA) comprising 0.2 mM myoinositol, 0.02 mM folic acid, 0.1 mM 2-mercaptoethanol, 200 U/ml recombinant IL-2, (Elabscience, USA), 12.5% fetal bovine serum (ATCC), and 12.5% horse serum (ATCC). The cells were grown at 37°C in a humidified 5% CO₂ incubator (Galaxy 170 R, New Brunswick, Scotland).

Flavonoids (apigenin, luteolin, quercetin)

Apigenin, luteolin, and quercetin (Sigma-Aldrich, Saint-Quentin-Fallavier) (Fig. 1) were dissolved in 100 % dimethylsulfoxide (Merck, Germany) at a 1,000 μ g/ml stock concentration. The flavonoid samples were then serially diluted with the α -MEM to a final working concentration. All the samples were freshly prepared and diluted before the experiment began.

Cytokine assay

To examine the effects of the three flavonoid compounds on NK cells cytokine productions, NK-92 cells (1×10^7 cells/ml in each well) were treated with various concentrations of the three flavonoid compounds; 12.5 and 25 μ g/ml concentrations were chosen for apigenin and luteolin whereas 25 and 50 μ g/ml concentrations were used for quercetin, respectively. The optimal flavonoid concentration used in this study was obtained during our previous research experiments (Oo *et al.*, 2020). For NK cell stimulation, LPS (*E. coli*, O55:B5, Sigma) at a concentration of 2.5 μ g/ml was introduced. The samples were maintained for 24 h at 37°C in a humidity chamber with 5% CO₂ and 95% air. The supernatant from the cultured cells was gathered and stored at -80°C for cytokine assays after the samples were centrifuged.

An enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of type-1 cytokines IFN- γ and IL-2, as well as type-2 cytokines IL-4 and IL-10 (Th1/Th2 Uncoated ELISA,

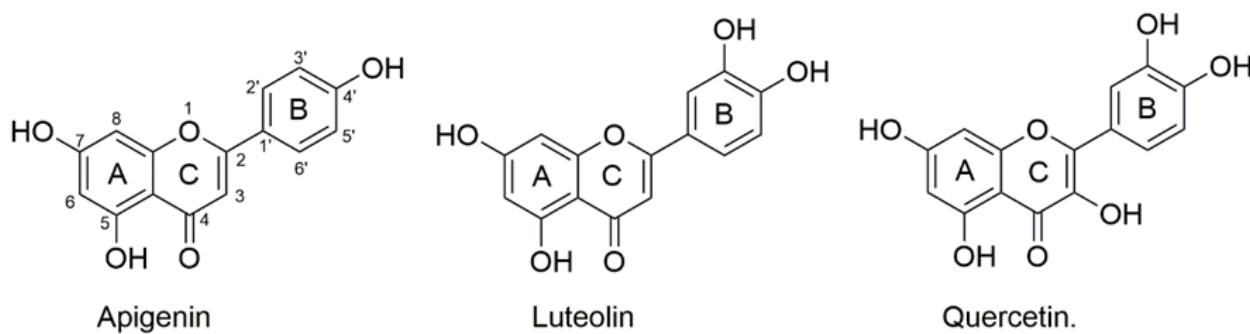


Figure 1. Structures of apigenin, luteolin, and quercetin. (Diagram adapted from Panche *et al.*, 2016.)

Invitrogen, Thermo Fisher Scientific, Austria) according to the established method (Ku and Lin, 2013). In brief, the ELISA plate (Corning Clear Flat Bottom Polystyrene High Bind Microplate, Life Sciences) was layered with 100 µl/well of capture antibody (as mentioned in the protocol). The samples were again secured and incubated for 24 h at 4°C. After washing, 200 µl of the diluent was added to the wells and incubated for 60 min at room temperature. After adding 100 µl/well of the samples to the appropriate wells, they were incubated at room temperature for 2 hours. Then, 100 µl/well diluted detection antibody was added to each well after washing the plate and incubated at room temperature for 1 hour. Lastly, streptavidin-HRP, a tetramethylbenzidine solution, and a stop solution were added as mentioned in the manufacturer's guidelines. The absorbance values were measured at 450 nm.

Statistical analysis

The data were presented as mean ± standard deviation of three measurements with duplicate samples. Statistically significant differences between untreated NK cells and NK cells treated with different concentrations of the three flavonoid compounds were examined using one-way analysis of variance, preceded by Dunnett's multiple comparisons test using the GraphPad Prism 6.0 application. At $p < 0.05$, differences were considered statistically significant. The following significance levels were reported: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

RESULTS

As shown in Figure 2, the ELISA result indicated that NK cells' IFN-γ secretion was significantly elevated with luteolin 25 µg/ml concentration ($p = 0.0029$); however, no significant

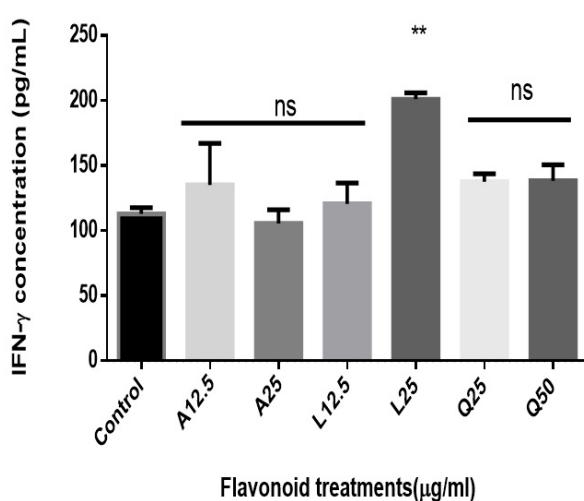


Figure 2. The effect of apigenin, luteolin, and quercetin on the interferon-gamma secretion of NK-92 cells. The values displayed are the mean (standard deviation) of three separate observations. * $p < 0.05$ and ** $p < 0.01$ mean the significant difference between control and treated samples. (A = apigenin, L = luteolin, Q = quercetin, and ns = no significant.)

changes were seen with low-dose luteolin (12.5 µg/ml). Similarly, IFN-γ production by NK-92 cells pretreated with flavonoids apigenin and quercetin shows no significant changes ($p > 0.05$).

The IL-2 secretion by NK cells was significantly increased by both luteolin and quercetin at all concentrations, but no changes were noted by apigenin, as illustrated in Figure 3. NK cells administered with luteolin 25 µg/ml exhibit the highest stimulation of IL-2 secretion ($p < 0.0001$) followed by quercetin 50 µg/ml dose ($p = 0.0003$) and 25 µg/ml dose ($p = 0.0017$). In contrast, no significant IL-2 secretion was noted with apigenin-treated NK cells.

In the case of NK cell IL-4 and IL-10 secretions, as seen in Figures 4 and 5, no significant changes were observed with all three flavonoid compounds at different doses. In other words, flavonoids exhibit no considerable alteration in NK cell IL-4 and IL-10 secretions.

DISCUSSION

Flavonoids and other polyphenolic compounds found in many foods were shown to improve the function of cells of innate immunity, such as NK cells (Di Renzo et al., 2019). Furthermore, plant-derived immunomodulators were shown to influence immune function activity by dynamically regulating informational substances such as cytokines. This interaction explains how herbs impact the immune system and other tissues. Cytokines are soluble proteins produced by various lymphocytes and play an essential role in immunomodulation. Cytokines are classified into type-1 cytokines (T helper 1 derived cytokines) and type-2 cytokines (T helper 2 derived cytokines) (Nworu et al., 2012; Spelman et al., 2006). NK cells are categorized as the cell of innate immunity because, without prior exposure, they react promptly, release chemical effector cytokines, and destroy diseased or altered cells (Zhang and Huang, 2017).

This research expresses an effort to identify and exploit bioactive flavonoids as potential agents for modulating cytokine secretion. This study found a significant rise in IFN-γ secretion by luteolin-treated NK cells (25 µg/ml). However, the other two flavonoids, apigenin and quercetin, as well as low-dose luteolin, did not produce the same results. IFN-γ is a soluble informational molecule released primarily by NK-T cells and NK cells during innate immunity, as well as by other lymphocytes after antigen-specific immunity has been established (Schoenborn and Wilson, 2007). As a result, it participates in an immunoregulatory, antiviral, and anticancer activity, as well as intensifying the function of NK cells (Schroder et al., 2004).

Likewise, luteolin and quercetin treatment increased NK cell IL-2 secretion, but apigenin did not. The highest IL-2 secretion was observed in NK cells treated with 25 µg/ml luteolin. Thus, luteolin showed the most vigorous stimulation of both IFN-γ and IL-2 among the three flavonoids. It is assumed that the differences in structural and functional properties of apigenin, luteolin, and quercetin may affect their activities against NK cells. Although all three bioactive compounds are flavonoids, they fall under different subclasses; for example, apigenin and luteolin are flavones,

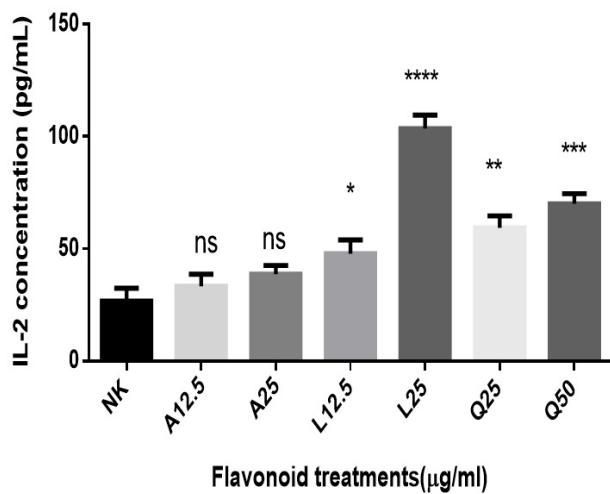


Figure 3. The effect of apigenin, luteolin, and quercetin on the IL-2 secretion of NK-92 cells. The values displayed are the mean (standard deviation) of three different findings. * $p < 0.05$ and ** $p < 0.01$ mean the significant difference between control and treated samples. (A = apigenin, L = luteolin, and Q = quercetin.)

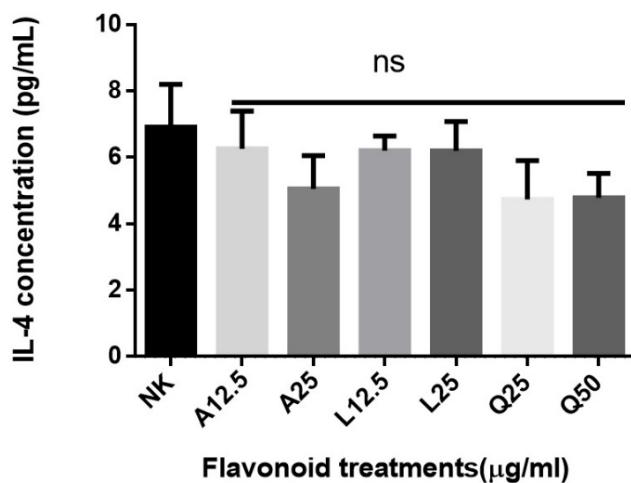


Figure 4. The effect of apigenin, luteolin, and quercetin on the IL-4 secretion of NK-92 cells. The values displayed are the mean (standard deviation) of three different findings. (ns = no significant, A = apigenin, L = luteolin, and Q = quercetin.)

whereas quercetin is under the flavonol subclass. Furthermore, the immunomodulatory and cytoproliferative effects of plant secondary metabolites are apparently driven by the presence of the hydroxyl side chains attached to these biomolecules, as well as the modulatory action of an electron transmitting system or enzyme mechanism, which activates immune cells (Manosroi *et al.*, 2003).

According to previous research data, IFN- γ production by innate lymphocytes is interconnected with NK cell IL-2 (Okoye *et al.*, 2016). The IL-2 level required to stimulate NK cells IFN- γ production is achieved significantly with luteolin 25 $\mu\text{g}/\text{ml}$ concentration but not with quercetin. We observed a nearly fourfold rise in IL-2 production by luteolin 25 $\mu\text{g}/\text{ml}$ treatment. Although quercetin-treated NK cells increase IL-2 secretion to

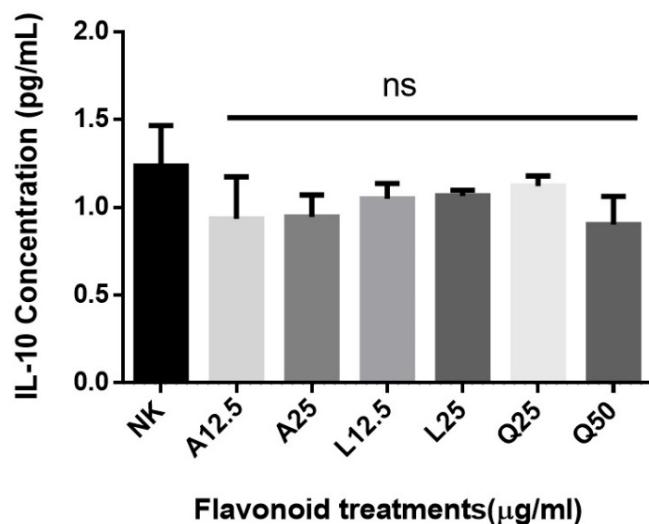


Figure 5. The effect of apigenin, luteolin, and quercetin on the IL-10 secretion of NK-92 cells. The values displayed are the mean (standard deviation) of three different findings. (ns = no significant, A = apigenin, L = luteolin, and Q = quercetin.)

some extent, that amount is insufficient to bind and stimulate IL-2 receptors and subsequent production of IFN- γ . Our findings agreed with a prior report conducted by Ismail N and colleagues, who documented that NK cell IL-2 and IFN- γ secretion significantly rose with luteolin treatment (Norzila *et al.*, 2018).

Although NK cell type-2 interleukin secretion seemed to decline slightly, we observed that there was no substantial change in both IL-10 and IL-4 secretions of flavonoid-treated NK cells compared to untreated control ($p > 0.5$). Furthermore, the findings depicted that the three flavonoids had no significant effect on type-2 cytokine secretion. However, other studies have found that the use of flavonoids has a different effect, with apigenin and luteolin dramatically reducing immune cell IL-4 production (Hirano *et al.*, 2004). Another study also demonstrated that quercetin greatly decreases the expression of the IL-4 gene and also the secretion from blood lymphocytes (Nair *et al.*, 2002). The nature of the cells studied and the dose of the flavonoids used could be the cause of the disparity in results. Other studies used basophils and peripheral blood mononuclear cells, whereas we used the NK-92 cell line. Furthermore, the flavonoids tested in our study are less concentrated than the flavonoids tested in the other two studies mentioned above.

As a result, the flavonoids luteolin and quercetin are thought to exert immunomodulatory effects via NK cell type-1 cytokine secretion rather than type-2. Furthermore, IL-4 and IL-10 significantly affect humoral immunity more than innate immunity, with NK cells falling into the latter category. Previous studies came to similar conclusions (Azadmehr *et al.*, 2016).

In the case of apigenin, rather than the four interleukins tested in our study, it may impose NK cell-mediated cytotoxic effects through the processing of other cytokines. Previous studies reported the mechanism of apigenin using the different molecular mechanisms in immunomodulation and in killing cancer, such as upregulation of NK receptors and activation of the intracellular kinases and other types of cytokines for its mechanism of killing cancer (Che *et al.*, 2020; Hougee *et al.*, 2005). However,

NK cell cytokine production is linked to NK cell membrane receptor activation or inhibition and various intracellular kinases activation. Therefore, the likely intracellular mechanisms pointing to the insignificant changes in NK cells IL-4 and IL-10 are still poorly understood and are being studied further, emphasizing NK cell receptors and intracellular kinases. Nonetheless, the present study's immunomodulatory effect of the three flavonoids on NK cells has shortcomings because there are far more regulating factors that could be taken into account; for example, other cytokines, NK cell membrane receptors, and intracellular kinases should be thought of, which are suggested for future study.

To conclude, our study revealed that luteolin and quercetin modulate immune response balance to type-1/Th-1 cytotoxicity without significantly affecting type-2 cytokine production. Moreover, luteolin-treated NK cells demonstrated promising outcomes in modifying cytokine production and maintaining a healthy dynamic equilibrium of type-1 and type-2 protein cytokines. The mechanisms involved in luteolin's immunomodulatory actions could be a potential platform for establishing novel flavonoid-based nutraceutical products for cancer immunotherapy. Overall, luteolin's immunomodulatory effect holds the possibility of being a more secure immunotherapy for cancer henceforward.

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CONFLICTS OF INTEREST

The researchers do not have any conflicts of interest. As a result, the authors are solely accountable for the paper's outcome.

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AUTHORS CONTRIBUTIONS

Prof NS, Dr. LHMA, and Dr. NMN conceptualized and designed the research work and made a contribution to manuscript preparation and formatting. Dr. AMO and Dr. OML carried out laboratory work, data collection and analysis, and the draft manuscript preparation, referencing, and corrections. Prof USMR was in charge of the manuscript's comprehensive review and proofreading. In addition, all lecturers made significant contributions to the literature review. The final report of the article was accepted by all of the authors.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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