Immunomodulatory effects of flavonoids: An experimental study on natural-killer-cell-mediated cytotoxicity against lung cancer and cytotoxic granule secretion profile

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Abstract

Background: A new approach involving immune-cell-mediated cancer therapy has been adopted extensively for the sake of lung cancer treatments by utilizing natural killer (NK) cells. NK cell activity can be enhanced with certain agents, and among them are flavonoids. Thus, this study was conducted to investigate the immunomodulatory roles of apigenin, luteolin and quercetin on NK cell activity against lung cancer cells and on the secretions of perforin and granulysin profile.

Methods: The NK-92 cells were grown in complete α-Minimum Essential Medium (MEM). NCI-H460 lung cancer cells were cultured in Roswell Park Memorial Institute 1640 media. NK cell activity against lung cancer cells were done using MTT(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The secretions of perforin and granulysin profiles were then analysed using enzyme-linked immunosorbent assay.

Results: Apigenin, luteolin and quercetin significantly increased the NK-cell-mediated cytotoxic activity against lung cancer cells at concentrations 12.5 µg/ml and 25µg/ml (P < 0.001). The secretion levels of perforin and granulysin from NK cells were also significantly enhanced with apigenin and luteolin treatment but not with quercetin.

Conclusions: All three flavonoid compounds possessed some significant immunomodulatory actions on NK cell cytotoxic activity and granule secretion profiles towards lung cancer therapy.

Keywords

Flavonoids, NK cells, lung cancer, cytotoxic granules

Introduction

Global lung cancer incidence is drastically increased and has recently reached the highest among all types of cancer. The World Health Organization estimated that the incidence of lung cancer was 11.6% of nearly 18 million new cancer cases diagnosed in 2018 (highest among all cancer), with the highest mortality rate of 18.4%. In Malaysia, more than 4600 new lung cancer cases were diagnosed in 2018, and the majority died, despite being treated with radiotherapy, chemotherapy, surgery or combined methods.1

Immune-cell-mediated cancer therapy is a new area of interest, and many scientists have been extensively studying the role of cytotoxic immune cells in the prevention and treatment of cancer. One of the promising cells to fight against the cancer cell is natural killer (NK) cells, which also kill various microbes and transformed cells of the body. The NK cell is also known as a cytotoxic lymphocyte of the innate immune system and comprises approximately 10–15% of the blood lymphocytes. They recognise and then spontaneously kill ‘stressed’ cells such as tumour- or viral-infected cells.2
Interestingly, NK cells kill virus or cancer cells either by direct cytotoxicity or initiation of apoptosis or both. Perforin, granulysin and granzyme are three cytolytic proteins stored inside NK cells. The destructive properties of these proteins to the targeted cells and leading to their lysis are the crucial role in the NK-cell-mediated cytotoxicity against cancer. Perforin destroys target cells by making pores and allows the entry of other proteases to induce lysis and apoptosis.\(^3\)\(^5\) Granulysin is a membrane-destructing protein released by innate lymphocytes such as NK and cytotoxic T cells (CD8) when they are activated. It destroys target cells by creating a hole in their membranes and then it kills them.\(^6\)

Long ago, researchers had continuously studied various botanical plants and their effects on the activity of NK cells against malignant diseases. Many scientists have endorsed the role of flavonoids in health and diseases. Flavonoids are the abundant phytonutrients found naturally in various fruits and vegetables. Based on their chemical structure, flavonoids are sub-classed into flavone, flavonol, flavonone, isoflavanonoid and anthocyanin.\(^7\) The two flavones apigenin and luteolin are well known for their immunomodulatory activity.\(^8\) Quercetin, a subclass of the flavonoid group, showed significant impact on cytotoxic immune cells.\(^9\) However, there have been limited studies done on the effect of apigenin, luteolin and quercetin of flavonoid subclasses.

Despite having various therapeutic measures to manage lung cancer, its incidence and mortality rates are still extremely high. It is time to consider alternative measures such as plant-derived bioactive compounds to fight against cancer and its mortality rate. We first reported the role of flavonoids derived bioactive compounds to fight against cancer and its lung cancer, its incidence and mortality rates are still extremely limited studies done on the effect of apigenin, luteolin and quercetin of flavonoid subclasses.

The experiments were conducted under strict aseptic conditions using a biological safety cabinet (ESC II series, Erla Technologies). The killing activity of NK cells treated by apigenin, luteolin and quercetin were evaluated using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described.\(^10\) Briefly, 100 µl of 5 x 10\(^6\) cells/ml NK cells were pre-incubated for 24 hours and subsequently treated with different concentrations of serially diluted flavonoids (6.25, 12.5, 25, 50 and 100 µg/ml, respectively). After 24 hours, NK cells were washed with RPMI media and seeded in a 96-well plate. Then, 100 µl aliquot of target NCIH-460 lung cancer cells (5 x 10\(^6\) cells/ml) (effector:target ratio 1:1) were added to each well in a 96-well plate and incubated for four hours at 37°C in a 5% CO\(_2\) humidified incubator (New Brunswick, Scotland). Subsequently, 20 µl of MTT (5 mg/ml) were added to the cells in the dark and incubated for three hours, covered with aluminum foil. After incubation and removal of media, 50 µl of DMSO was added to each well to dissolve the formazan crystals formed and incubated for another 15 minutes in the incubator. The absorbance was read at a wavelength of 570 nm as measurement wavelength using the Tecan enzyme-linked immunosorbent assay (ELISA) micro-plate reader (finite M200PRO, TECAN, Switzerland). NK cell activity was calculated according to a formula described previously:\(^15\)

\[
\text{NK activity (}) = 100\% \times (\text{ODT} - (\text{ODS} - \text{ODE})) / \text{ODT}
\]

where ODT is the optical density value of the target cells (NCIH-460 lung cancer cells) control, ODS is the optical density value of the test samples and ODE is the optical density value of the effectors cells (NK-92 cells) control, target cells’ control (NCIH-460 lung cancer cells).

### Assessment of perforin and granulysin secretion

The level of perforin and granulysin secretion from the treated NK cells was determined using pre-coated ELISA according to a previous method.\(^1\) In brief, NK cells (1 x 10\(^7\) cells/ml) were incubated with three flavonoid compounds in non-toxic concentrations (12.5 and 25 µg/ml for apigenin and luteolin, and 25 and 50 µg/ml for quercetin) over 24 hours at 37°C in a humidified incubator. After incubation, plates were centrifuged, and supernatant was collected. The perforin and granulysin (Human Perforin ELISA Kit-ab46068, Human Granulysin ELISA Kit-ab13787, Abcam, USA) levels were
measured using an ELISA reader at 450 nm wavelength according to the manufacturer’s protocol.

**Statistical analysis**

The results were expressed as mean ± standard deviation (SD) of three independent experiments in triplicate samples. Statistical differences between the control and the flavonoid compounds with different concentrations to the cell line were analysed using one-way analysis of variance, followed by Dunnett’s multiple comparison tests using the GraphPad Prism 6.0 program. Differences were considered to be statistically significant at $P < 0.05$. Significance levels were reported as follows: $^* p < 0.05$, $^{**} p < 0.01$, $^{***} p < 0.001$ and $^{****} p < 0.0001$ mean significant difference between control and treated samples. $ns$: not significant.

**Results**

The effect of apigenin, luteolin and quercetin concentrations on NK-cell-mediated cytotoxicity against lung cancer cells

The results revealed that all three flavonoids induce marked elevation of NK-cell-mediated cytotoxicity against lung cancer cells (Figure 1). The cytotoxic activity of NK cells was stimulated by all three flavonoids in a dose-dependent manner. The data illustrated that each compound imparted significant effect at all tested concentrations except 6.25 µg/ml for quercetin and 100 µg/ml for apigenin. The highest activity was achieved at 12.5 and 25 µg/ml of apigenin and luteolin (150 and 140%, respectively) compared to the control value of 48%; however, quercetin required 50 µg/ml to obtain the maximal activity. It is also observed that the NK cell killing effect declined as the doses of the three flavonoids were increased to a higher concentration (100 µg/ml).

**Figure 1.** The effects of various concentrations of apigenin, luteolin and quercetin on NK-cell-mediated cytotoxicity against lung cancer in vitro. NK cells were cultured with different doses of apigenin, luteolin and quercetin for 24 hours before NCI-H460 target cells were added (at expected 1:1 effector:target ratio, NK cells act as effector cells, whereas lung cancer cells are known as target cells). NK cell activity was then measured using an MTT assay. Values shown are mean ($±$ SD) percentage cytotoxic activity from three different observations.

**Figure 2.** The effect of apigenin, luteolin and quercetin on NK-92 perforin secretion profile. NK cells were pre-treated with different doses of three flavonoids (12.5 and 25 µg/ml for apigenin and luteolin, and 25 and 50 µg/ml for quercetin) overnight. Supernatant was collected, and perforin analysis was done using the sandwich ELISA method. Values shown are mean ($±$ SD) from three different observations. $^* p < 0.05$ means significant difference between control and treated samples.

A: apigenin; L: luteolin; Q: quercetin.
The effects of apigenin, luteolin and quercetin on NK-92 perforin secretion profile

As shown in Figure 2, the perforin secretion increased with all tested concentrations of the three flavonoids compared to the untreated control. However, for apigenin and luteolin, especially at 12.5 µg/ml concentration, both flavonoids imparted significant effect. No significant differences were detected with 25 µg/ml tested doses of both apigenin and luteolin as well as with all tested doses of quercetin.

The effects of apigenin, luteolin and quercetin on NK-92 granulysin secretion profile

The ELISA results demonstrated that the NK cell protein granulysin secretion was obtained after treated with different doses of three flavonoids. In particular, the significant production of granulysin was found, especially with a 25 µg/ml dose of apigenin and both 12.5 and 25 µg/ml concentrations of luteolin (Figure 3). On the other hand, quercetin exerted no significant difference in NK cell granulysin secretion at all tested concentrations compared to the untreated control. The maximal NK cell granulysin secretion was achieved with 12.5 µg/ml of luteolin (28 pg/ml) compared to the control group (10 pg/ml).

Discussion

Lung cancer is recently considered as the deadliest among all types of cancer. NK-cell-based immunotherapy is one of the promising ways of treating various types of cancer. Naturally occurring plant-based flavonoids have been extensively studied for their role in immunity. There are several studies that have shown the ability to increase the activity of NK cells by naturally occurring compounds. Phenolic compounds such as apigenin, luteolin and quercetin are potential therapeutic agents for immune system modulation and cancer prevention. With reference to these studies, the present research was conducted to investigate the roles of apigenin, luteolin and quercetin in the cytotoxic activity of NK cells against lung cancer cells.

In this study, the cytotoxic activity of NK cells against lung cancer was significantly enhanced after being treated with a series of concentrations of apigenin, luteolin and quercetin as compared to the untreated control. Apigenin and luteolin showed a marked increase in the killing of cancer cells, especially at low concentrations (12.5 and 25 µg/ml), whereas quercetin required a higher dose (50 µg/ml) to achieve the optimal killing effect. This is likely due to the nature of flavonoid, since apigenin and luteolin belong to the flavone group, whereas quercetin is flavonol. The alteration in chemical structure and the number of hydroxyl groups present in each flavonoid as shown in figure 4 react differently in the activation of NK cells. In fact, the functional differences in biological effects of flavonoids are critically related with their chemical constitution fragment, side-chain variation and substituent groups, although they act on the same target.

Surprisingly, the gradual decline in the killing effect of pre-treated NK cells was noted in all three flavonoids when increasing their concentrations to a higher dose. In other words, the higher the dose of flavonoids, the lesser the NK cell killing effect was observed. Assumably, a higher dose of flavonoids might disturb the proliferation, activity and metabolism of NK cells or their killing mechanisms. The cell activity and metabolism in vitro become slower when proliferation is
excessive, or cells are packed in the cultured plate, which leads to contact inhibition.\textsuperscript{15} A study conducted in Tunisia also reported similar findings, where the authors documented that the higher killing activity of NK cells was found in low doses, but the effect declined when the flavone concentrations were increased.\textsuperscript{8} Similarly, another flavonoid class—namely, resveratrol—also favoured NK cell cytotoxicity at low doses; however, a dose-related inhibition of lytic activity was endorsed at high resveratrol concentrations.\textsuperscript{16} In the case of luteolin, it is documented that the killing of NK cells against human myeloid leukaemia K562 cells was significantly increased after treated with luteolin. The authors also demonstrated that the enhanced killing was due to the increased secretion of cytokine IL-2 and interferon.\textsuperscript{17}

Arguably, the results obtained for quercetin treatment on NK cell activity were reported equivocally. Yu et al. reported that quercetin enhanced NK cell activity in BALB/c mice administered with WEHI-3 leukaemia cells and subsequently treated with quercetin. NK cell activity of leukocytes was increased and resulted in enhanced killing activity, which was demonstrated with YAC-1 target cells.\textsuperscript{18} In contrast, the flavonoid quercetin was found to inhibit the killing activity of NK cells in circulating lymphocytes from human donors at high concentrations (100 µmol/L). Decreased cytosis was observed and suggested to be caused by the inhibition of Ca\textsuperscript{2+} channels and Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity by quercetin.\textsuperscript{19} In addition, daily quercetin supplementation for 12 weeks significantly increased plasma quercetin levels but had no influence on measures of innate immune function in adult females.\textsuperscript{20} However, we observed that NK cell killing activity towards lung cancer was significantly elevated after quercetin treatment compared to the untreated control. The nature of flavonoid compound, different cancer cell lines used in the study and the source of NK cells obtained might have contributed to the ambivalent findings.

The killing mechanisms of NK cells to destroy their target include cytotoxic granule secretion, cytokine production and antibody-dependent cellular toxicity. We examined the effects of all three flavonoids on NK cell perforin and granulysin secretion using the sandwich ELISA method. The perforin secretion by NK cells was significantly elevated after being treated with apigenin and luteolin in this study. This cytotoxic protein release is more significant when treating with a low dose of apigenin and luteolin (12.5 µg/ml). However, quercetin-treated NK cells have no significant impact on perforin release compared to normal control. Previous studies endorsed the effect of flavonoids on NK cell perforin secretion. Hou et al. reported that total flavonoids of Hippophae rhamnoides-activated NK cells enhance cytotoxic killing against K562 cancer cell line. The authors explained that the mechanisms of the flavonoid on NK-cell-mediated toxicity are due to their favoured expression of perforin and granzyme cytolytic proteins as well as up-regulation of various cytokines.\textsuperscript{21} However, a study documented that quercetin treated T-cell-mediated killing towards human colon cancer cells by increasing the expression of perforin, granzyme and interferon.\textsuperscript{9} Their findings were not consistent with our observations since we found insignificant changes in quercetin-induced NK cell perforin secretion. This is likely due to the difference in the nature of the cells. Although both NK cells and T cells are cytoxic lymphocytes, they differ in some phenotypical and chemical properties, as well as in the presence of receptors in their membranes.\textsuperscript{22} Quercetin may bind and modulate differently in these different lymphocytes.

In this study, we found that apigenin- and luteolin-treated NK cells enhanced the release of granulysin compared to the untreated control, but the same response was not found with quercetin. The two flavones showed a significant release of cytotoxic granule secretion compared to flavonol quercetin in this study. Luteolin increases NK cell granulysin secretion at both 12.5 and 25 µg/ml concentrations, while apigenin-induced secretion of this protein is significant only with 25 µg/ml. Interestingly, low doses of apigenin and luteolin (12.5 µg/ml) showed stronger effects towards perforin release; however, in the case of granulysin, they displayed different effects. Apigenin required a higher dose (25 µg/ml) to activate significant secretion of NK cell granulysin. Once activated, NK cells release cytoplasmic granules containing proteins such as perforin, the saposin-like family member granulysin and serine proteases called granzymes, like granzyme B, to cleave, for example, several pro-caspases, which are then able to trigger apoptosis in the target cell.\textsuperscript{23,24} The synthesis and the exocytosis of these cytotoxic granules are influenced by the activation of NK cell receptors, cytokine production and different signalling pathways, as well as the influence of various plant-derived nutrients.\textsuperscript{9,21,25,26} It is postulated that apigenin and luteolin would modulate the synthesis and release of NK receptors and cytokine and cytotoxic granule release. Our ongoing experiments on NK cell receptor and cytokine secretion profile by apigenin, luteolin and quercetin would be able to link to NK-cell-mediated cytotoxicity against lung cancer.

In addition, the presence of double bond and the difference in the positions of the hydroxyl group may play a role in the binding to NK cell receptors and subsequent activation of NK cell cytotoxic granule expression as well as its release. Hydroxyl
(-OH) groups are present by luteolin and apigenin in their backbone, and it was suggested that these -OH may be involved in immunomodulatory activities since luteolin, which contains two hydroxyl groups at ring B, demonstrated stronger immunomodulatory properties than apigenin, which has only one hydroxyl group.11,12 NK cell cytotoxic granule secretion is enhanced by increasing exocytosis, which is due to increasing intracellular calcium secretion via tyrosine-kinase-mediated or other calcium-dependent kinase activation.27,28 Most likely, these flavonoids may either directly increase cytosolic calcium content or activate the kinase or both.

Both perforin and granulysin secretion were not significantly increased after quercetin treatment. The molecular weight (MW) of quercetin (MW = 302) is the highest among the three flavonoids (MW = 270 for apigenin, MW = 286 for luteolin), which could possibly affect the NK cell granule secretion.29 Systemic bioinformatics analysis of flavonoids carried out by researchers from Shanghai documented that different structural and physico-chemical properties as well as variation in MW of different flavonoids may alter their functional properties.30 Further experiments are currently in progress to determine the different intracellular mechanisms responsible for the activation of NK-cell-mediated cytotoxicity against lung cancer.

Conclusion
In conclusion, NK-92 cell-mediated cytotoxicity against lung cancer cells was observed with all three flavonoids: apigenin, luteolin and quercetin. Apigenin- and luteolin-treated NK cells potentiate the secretion of cytotoxic granules for their NK-cell-mediated cytotoxicity against lung cancer; whereas quercetin showed no similar effect. Quercetin might portray different intracellular mechanisms in activating NK cells against lung cancer. Based on our findings, it could be deduced that apigenin, luteolin and quercetin could facilitate in NK cell-based cancer therapy. It is meritorious to further investigate these three flavonoids and their effect on immunomodulation in vivo.

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Authors’ contributions
Prof Nordin Simbak, Dr Lyana Hazwani and Dr Nasir Mat Nor designed and provided concepts for the research project, and contributed to manuscript preparation and editing. Laboratory work, data acquisition and data analysis were carried out by Dr Aung Myo Oo, Dr Ohn Mar Lwin and Ms Nor Zidah, who also contributed to the preparation of the draft manuscript, referencing and corrections. All lecturers contributed substantial amount on literature search.

Availability of data and materials
The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declaration of conflicting interests
The authors have no conflicts of interest to declare.

Ethical approval
Sultan Zainal Abidin University does not require ethical approval for reporting individual cases or case series.

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Informed consent
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