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Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

*Original Article*

# Bifunctional activity of tangeretin (5,6,7,8,4'-pentamethoxyflavone) in suppression of cell growth and gene expression of the superoxide-generating system-related proteins in U937 cells

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(Received September 6, 2022; Accepted September 12, 2022)

**ABSTRACT** — Polymethoxyflavones in which all hydroxyl groups are capped by methyl residues show high membrane permeability, metabolic resistance, and diverse biological activities such as cell growth inhibitory effects responsible for their anti-cancer activities. They are expected to be as biotherapeutic drugs for maintaining human health via contributing prevention and treatment of serious illnesses such as cancers, neurodegenerative diseases, inflammatory bowel diseases, lipid metabolism disorders, and so on. Human monoblastic leukemia U937 cell line has been used as an excellent *in vitro* cell model system for studying macrophage differentiation induced by various cell differentiation inducers such as all-*trans* retinoic acid (ATRA). In this study, we investigated the influences of two typical polymethoxyflavones, such as nobiletin and tangeretin, on the ATRA-induced superoxide anion ($O_2^-$)-generating ability of U937 cells. Nobiletin and tangeretin suppressed cell proliferation of U937 cells in a dose-dependent manner. At a concentration of 10 μM, tangeretin drastically brought about down-regulation of the ATRA-induced $O_2^-$-generating ability (to ~15% of ATRA-treated cells) although nobiletin showed moderate inhibitory effects on the ATRA-induced $O_2^-$-generating ability (to ~65% of ATRA-treated cells). Quantitative RT-PCR and immunoblotting revealed that tangeretin down-regulates the ATRA-induced $O_2^-$-generating ability via suppressing gene exprerssion levels of gp91-phox (mRNA: to ~75%, protein: to ~70% of ATRA-treated cells) and p47-phox (mRNA: to ~75%, protein: to ~40% of ATRA-treated cells) genes. These findings demonstrated that tangeretin shows not only the inhibitory effects on cell growth but also the strong suppressing effects on the ATRA-induced $O_2^-$-generating ability of U937 cells.

**Key words:** Nobiletin, Tangeretin, Superoxide, Growth inhibition, all-*trans* retinoic acid, Leukocyte

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## INTRODUCTION

Higher plants produce many kinds of phytochemicals as secondary metabolites that protect them from numerous environmental risks, e.g., UVB irradiation, toxicants and various microbial infections. Human beings consume various, diverse and abundant phytochemicals by eating vegetables and fruits in their daily diet. Since phytochemicals show diverse biological functions, they have been getting attention as health-promoting substances. Polymethoxyflavones belonging to phytochemicals show high membrane permeability and metabolic resistance caused by methylation of their hydroxyl groups, and diverse biological activities (Walle, 2007). Nobiletin (5,6,7,8,3',4'-hexamethoxyflavone) and tangeretin (5,6,7,8,4'-pentamethoxyflavone), two typical polymethoxyflavones, abundantly present in citrus peel and exhibit various biological functions such as anti-cancer (Walle, 2007; Meiyanto *et al*., 2012; Xiu *et al*., 2015; Goh *et al*., 2019), anti-neurodegenerative diseases (Braidy *et al*., 2017; Matsuzaki and Ohizumi, 2012), anti-inflammatory bowel diseases (Chang *et al*., 2021), lipid metabolism (Assini *et al*., 2013; Mulvihill *et al*., 2016), anti-lipopolysaccharide-induced inflammatory bone loss (Tominari *et al*., 2012), anti-oxidant (Singh *et al*., 2020) and up-regulation of cell-cell interactions (Brack *et al*., 2002). Therefore, these compounds are expected to play important roles in prevention and treatment of various diseases (Lai *et al*., 2015; Ahmed *et al*., 2021).

On the other hand, the $O_2^-$-generating ability of phagocytes is an important function in innate immune system. The $O_2^-$-generating system in phagocytes carries an electron from NADPH to molecular oxygen ($O_2$). This system needs a membrane heterodimer protein cytochrome $b_{558}$ (composed of the small [p22-phox] and the large [gp91-phox] subunits), three specific cytosolic proteins (p40-phox, p47-phox and p67-phox), and ubiquitous small G-protein Rac (Dagher and Pick, 2007; Panday *et al*., 2015). Any defect in p22-phox, gp91-phox, p47-phox or p67-phox brings about chronic granulomatous disease (CGD) where phagocytes cannot generate $O_2^-$ upon microbial infections. Consequently, CGD patients suffer from serious diseases due to recurrent bacterial and fungal infections. Therefore, the significance of the $O_2^-$-generating ability of phagocytes is emphasized by CGD.

As previous known, various agents including all-*trans* retinoic acid (ATRA) can differentiate human monoblastic leukemia U937 cells to macrophage-like cells (Harris and Ralph, 1985). Differentiation of U937 cells using ATRA has been used as a powerful model for *in vitro* studies on macrophage differentiation. While U937 cells generate a negligible level of $O_2^-$, the differentiated U937 cells can remarkably release $O_2^-$ into outside of their cell membane in response to various stimuli such as phorbol 12-myristate 13-acetate (PMA) (Kikuchi *et al*., 1994; Kikuchi *et al*., 2011). We have investigated the influences of phytochemicals on the ATRA-induced $O_2^-$-generating ability of U937 cells, for example, curcumin (Kikuchi *et al*., 2010), resveratrol (Kikuchi *et al*., 2018), chalcones (Kikuchi *et al*., 2019), sulforaphane (Akiyoshi *et al*., 2019), ellagic acid and urolithin A (Kikuchi *et al*., 2021a), hydroxyflavones (Kikuchi *et al*., 2021b) and L-theanine (Kikuchi *et al*., 2022). However, the understanding of the effects of polymethoxyflavones against the ATRA-induced $O_2^-$-generating ability of U937 cells is still unknown.

In this paper, we studied the effects of nobiletin and tangeretin on the ATRA-induced $O_2^-$-generating ability of U937 cells, and revealed that tangeretin strongly suppresses the ATRA-induced $O_2^-$-generating ability through down-regulating transcription of gp91-phox and p47-phox genes.

## MATERIALS AND METHODS

### Materials

Nobiletin, tangeretin (Tokyo Chemical Industry, Tokyo, Japan, their chemical structures are shown in Fig. 1), RPMI-1640 culture medium (Thermo Fisher Scientific, Waltham, MA, USA), PMA, ATRA, luminol (Sigma, St Louis, MO, USA), Diogenes-luminol chemiluminescence probes (National Diagnostics, Atlanta, GA, USA), fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), plasmocin (InvivoGen, San Diego, CA, USA), monoclonal anti-gp91-phox antibody, monoclonal anti-p47-phox antibody (BD Biosciences, San Jose, CA, USA), monoclonal anti-p22-phox antibody, monoclonal anti-p67-phox antibody, horseradish peroxidase-conjugated anti-goat immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p40-phox antibody (GeneTex, Irvine, CA, USA), monoclonal anti-β-actin antibody, monoclonal anti-$Na^+/K^+$-ATPase antibody (Abcam, Cambridge, UK), and horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (Promega, Madison, WI, USA) were obtained from companies indicated respectively.

### Cell culture, treatment with nobiletin or tangeretin in the presence of ATRA

Human monoblastic leukemia U937 cell line (RCB0435) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. U937 cells were grown in RPMI-1640 culture medium containing 10%

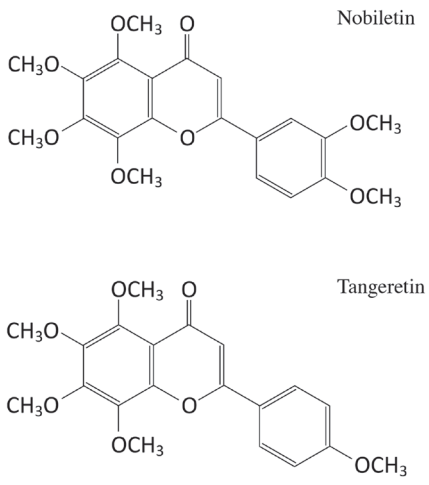


**Fig. 1.** Chemical structures of nobiletin and tangeretin.

FBS and 5 μg/mL plasmocin as described (Kikuchi *et al*., 2019). Cells (1.0 x $10^6$) in 5 mL of culture medium were incubated with nobiletin or tangeretin (up to 20 μM) in the presence of 1 μM ATRA up to 48 hr. The determination of the number of cells was carried out using a hemocytometer under a microscope.

### Measurement of $O_2^-$ generation

Measurement of $O_2^-$ generation was carried out by Lumat$^3$ LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany) using luminol and Diogenes-luminol chemiluminescence probes as described previously (Kikuchi *et al*., 2018).

### Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Cells (2.0 x $10^6$) in 5 mL of the culture medium were incubated with or without 10 μM nobiletin or 10 μM tangeretin in the presence of 1 μM ATRA at 37°C for 48 hr. Total RNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RT reaction was performed using a first strand cDNA synthesis kit ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan). RT-qPCR was carried out on the MyGo Mini S real-time PCR instrument (IT-IS Life Science, Cork, Ireland) using GeneAce SYBR qPCR mix α (Nippon Gene, Tokyo, Japan). PCR reaction and data analysis procedures were performed according to the attached manufactures' protocols. Sequence data of primers used for qPCR were listed in Supplementary Table S1 (Juhasz *et al*., 2009; Kitamoto *et al*., 2018). All qPCR data of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox genes were normalized to qPCR data of GAPDH gene.

### Immunoblot analysis

Cells (2.0 x $10^6$) in 5 mL of the culture medium were incubated with or without 10 μM nobiletin or 10 μM tangeretin in the presence of 1 μM ATRA at 37°C for 48 hr, disrupted in 100 μL of 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 2 mM EDTA and 1 mM PMSF. The cell lysates were divided into cytosolic fractions and membrane fractions by centrifugation. The cytosolic fractions were treated with 10% trichloroacetic acid, collected by centrifugation, dissolved in 100 μL of 0.5 M Tris-HCl (pH 6.8) containing 2.5% SDS, 10% glycerol, and 5% 2-mercaptoethanol, and heated at 100°C for 5 min. In contrast, the membrane fractions were suspended 50 μL of a solubilizing solution containing 9 M urea, 2% Triton X-100 and 5% 2-mercaptoethanol. After addition of loading buffer [0.5 M Tris-HCl (pH 6.8) containing 5% SDS, 20% glycerol], these protein samples were subjected to SDS-PAGE followed by immunoblot analysis. Data analyses were carried out using a luminescent image analyzer STAGE-5100. Human β-actin (for cytosolic fractions) and $Na^+/K^+$-ATPase (for membrane fractions) were used as controls (Kikuchi *et al*., 2019).

### Statistical analysis

Quantitative data are presented as averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated with Student's *t* test.

## RESULTS AND DISCUSSION

First, in order to investigate the effects of nobiletin and tangeretin on proliferation of U937 cells during differentiation induced by ATRA, the cells were treated with up to 20 μM of each compound in the presence of 1 μM ATRA. As shown in Fig. 2, both nobiletin and tangeretin suppressed cell proliferation of U937 cells in a dose-dependent manner. Similar results were also obtained in the absence of ATRA (Supplementary Fig. S1). Especially, these two polymethoxyflavones drastically inhibited proliferation of U937 cells at a concentration of 20 μM. These data suggested that there is a strong possibility that treatment with 20 μM of nobiletin and tangeretin may lead to exacerbation of the cell conditions. Therefore, in order to understand the influences of nobiletin and tangeretin on the ATRA-induced $O_2^-$-generating ability, we carried out the experiments at a concentration of 10 μM. Both nobiletin and tangeretin had little effect on the viability of the cells up to 10 μM in the presence of 1 μM ATRA (data not shown) although they moderate-

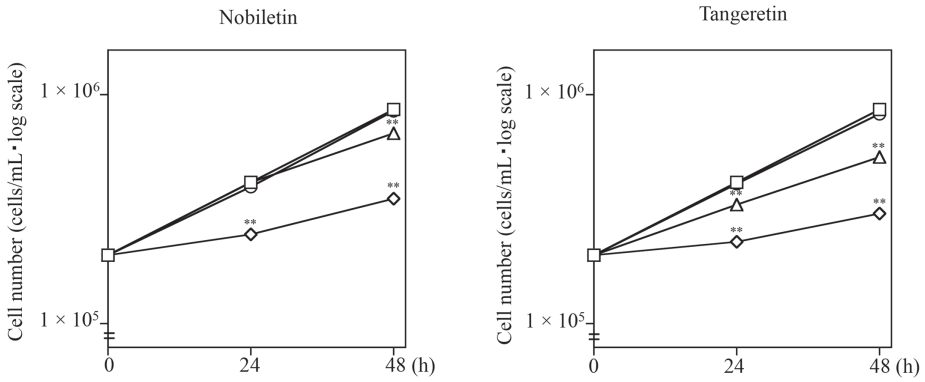


**Fig. 2.** Effects of nobiletin and tangeretin on cell growth of U937 cells. Cells (1.0 x $10^6$) in 5 mL of culture medium were incubated without (squares) or with 5 (circles), 10 (triangles) or 20 μM (rhombi) polymethoxyflavone (nobiletin or tangeretin) in the presence of 1 μM ATRA for up to 48 hr. Cell counts were carried out by a hemocytometer under a microscope at the indicated times in the graph. Data represent the averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated by Student's *t* test. **, $p < 0.01$ compared with the data of without polymethoxyflavones at each time point.

ly suppressed proliferation of the cells in the presence of 1 μM ATRA (Fig. 2).

Second, in order to know the influences of nobiletin and tangeretin on the ATRA-induced $O_2^-$-generating ability of U937 cells, the cells were treated with 10 μM of each reagent in the presence of 1 μM ATRA for 48 hr. As shown in Fig. 3, both nobiletin and tangeretin showed significant inhibitory effects on the ATRA-induced $O_2^-$-generating ability of U937 cells (nobiletin: to ~65% of ATRA-treated cells, tangeretin: to ~15% of ATRA-treated cells). These results demonstrated that these two polymethoxyflavones, especially tangeretin, have a strong inhibitory effect on the ATRA-induced $O_2^-$-generating ability of U937 cells.

Third, in order to investigate the effects of nobiletin and tangeretin on the transcription levels of five genes essential for the leukocyte $O_2^-$-generating system (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) in detail, we carried out RT-qPCR analysis. Total RNAs were prepared from ATRA-treated, ATRA plus 10 μM nobiletin, and ATRA plus 10 μM tangeretin-treated U937 cells. Quantitative data were indicated as percentages of control values obtained from ATRA-treated U937 cells (Fig. 4). Both nobiletin and tangeretin showed no effect on the transcription levels of p22-phox and p67-phox genes during co-treatment with ATRA. In constrast, the transcription levels of p40-phox were significantly up-regulated in ATRA plus nobiletin or tangeretin-treated U937 cells (nobiletin: to ~170% of ATRA-treated cells, tangeretin: to ~125% of ATRA-treated cells). While tangeretin significantly down-regulated the transcription levels of gp91-phox (to ~75% of ATRA-treated cells) and p47-phox (to ~75% of ATRA-treated cells) genes during co-treatment with ATRA, nobiletin showed unsignificant effect on those of gp91-phox and p47-phox genes during co-treatment with ATRA.

Finally, in order to examine the influences of nobiletin and tangeretin on the amounts of set of proteins essential for the $O_2^-$-generating system (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) during co-treatment with ATRA, immunoblot assay was carried out using antibody specific for each protein (Fig. 5A). Quantitative data were indicated as percentages of control values obtained from ATRA-treated U937 cells (Fig. 5B). The protein levels of p40-phox and p67-phox of ATRA plus polymethoxyflavone (nobiletin or tangeretin)-treated cells were unchanged compared to those of ATRA-treated cells. It is worthy of notice that the protein levels of p40-phox were not changed although its mRNA was increased in the presence of nobiletin or tangeretin (Fig. 4). The exact mechanism on the reverse observations on mRNA levels and protein levels were still not clear from the present set of experiments. On the other hand, the protein levels of p22-phox, gp91-phox and p47-phox were unchanged in the cells treated with ATRA plus nobiletin. In contrast, the protein levels of p22-phox (to ~60% of ATRA-treated cells) and gp91-phox (to ~70% of ATRA-treated cells) were significantly down-regulated in ATRA plus tangeretin-treated U937 cells. As mentioned above, p22-phox and gp91-phox proteins cooperatively assemble to form cytochrome $b_{558}$ heterodimer in the plasma membrane (Dagher and Pick, 2007; Panday *et al*., 2015). Our pre-

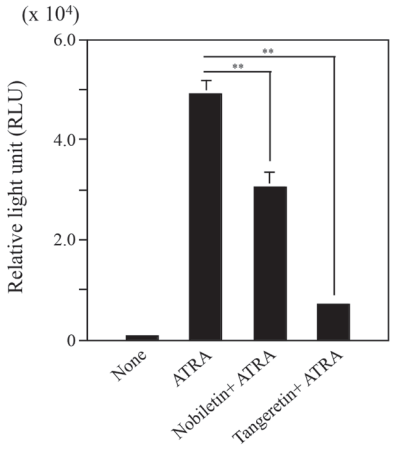


**Fig. 3.** Effects of nobiletin and tangeretin on the ATRA-induced $O_2^-$-generating activity of U937 cells. After cultivation with 10 μM polymethoxyflavone (nobiletin or tangeretin) in the presence of 1 μM ATRA for 48 hr, cells (1 x $10^5$ cells/mL) were stimulated with 200 ng/mL PMA at 37°C. PMA-induced chemiluminescence was measured at 10 min after stimulation using a Lumat$^3$ LB9508 luminometer as described previously (Kikuchi *et al*., 2018). Quantitative data represent the averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated by Student's *t* test. **, $p < 0.01$ compared with the data of ATRA-treated U937 cells.

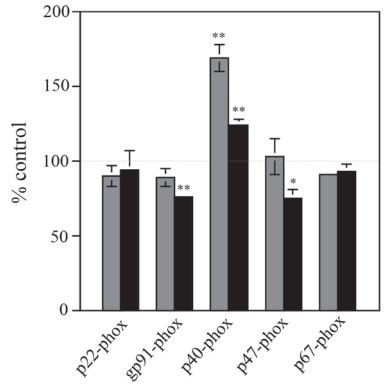


**Fig. 4.** Effects of nobiletin and tangeretin on the transcription levels of the $O_2^-$-generating system-related factors. The mRNA levels of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox were determined by RT-qPCR using total RNA extracted from ATRA-treated, ATRA plus 10 μM nobiletin-treated (shaded bars) and ATRA plus 10 μM tangeretin-treated (closed bars) U937 cells as described in "MATERIALS AND METHODS". All qPCR data of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox genes were normalized to qPCR data of GAPDH gene. Quantitative data are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. *, $p < 0.05$, **, $p < 0.01$ compared with the data of ATRA-treated U937 cells.

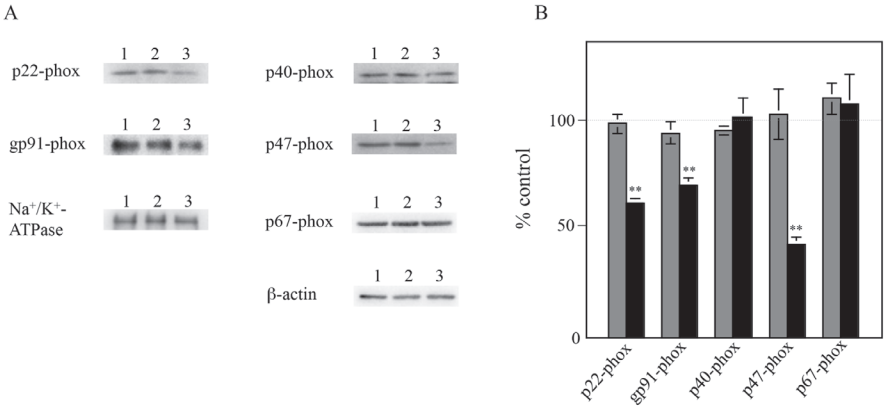


**Fig. 5.** Effects of nobiletin and tangeretin on the protein levels of the five $O_2^-$-generating system-related factors. (A) Typical immunoblot profiles. Membrane (for p22-phox and gp91-phox) and cytosolic (for p40-phox, p47-phox and p67-phox) fractions were prepared from ATRA-treated (lane 1), ATRA plus 10 μM nobiletin-treated (lane 2) and ATRA plus 10 μM tangeretin-treated (lane 3) U937 cells, and protein levels of the five $O_2^-$-generating system-related factors were determined by immunoblot analysis. Human $Na^+/K^+$-ATPase (for membrane fractions) and β-actin (for cytosolic fractions) were used as controls. (B) Quantitative data of immunoblot analysis. Data of ATRA plus 10 μM nobiletin-treated (shaded bars) and ATRA plus 10 μM tangeretin-treated (closed bars) U937 cells are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. **, $p < 0.01$ compared with the data of ATRA-treated U937 cells.

vious reports showed that the expression level of gp91-phox protein limits the $O_2^-$-generating activity of U937 cells (Kikuchi *et al*., 1994), and revealed that the amounts of p22-phox protein may tend to depend on those of gp91-phox in U937 cells (Kikuchi *et al*., 2021a, 2022). Moreover, the protein levels of p47-phox were also down-regulated in the cells treated with ATRA plus tangeretin (to ~40% of ATRA-treated cells). Taken together, our data showed that tangeretin suppressed the transcription levels of gp91-phox and p47-phox genes, resulting in remarkable decrease of cytochrome $b_{558}$ and p47-phox proteins, so that the ATRA-induced $O_2^-$-generating ability was down-regulated in U937 cells. However, the degree of inhibition of the ATRA-induced $O_2^-$-generating ability (to ~15% of ATRA-treated cells) seems to be not consistent with degree of decreases of p22-phox (to ~60% of ATRA-treated cells), gp91-phox (to ~70% of ATRA-treated cells) and p47-phox (to ~40% of ATRA-treated cells) proteins. On the other hand, nobiletin moderately inhibited the ATRA-induced $O_2^-$-generating ability of U937 cells (to ~65% of ATRA-treated cells) although it showed no effect on the levels of transcription of gp91-phox and p47-phox genes. These results suggested that these two methoxyflavones may have some negative effects on the ATRA-induced $O_2^-$-generating ability of U937 cells other than inhibitiory effect of transcription of gp91-phox and p47-phox genes. As shown in Fig. 2, both nobiletin and tangeretin showed the growth suppression effect on U937 cells in the presence of ATRA. It seems that such growth suppression effects of nobiletin and tangeretin on U937 cells may involved in the negative effects on the ATRA-induced $O_2^-$-generating ability of U937 cells. Of course, further studies are still required to understand the exact mechanism.

As is well known, many flavonoids including polymethoxyflavones have anti-cancer activities due to their inhibitory effects on cell growth (Manthey *et al*., 2001; Manthey and Guthrie, 2002). From this investigation, it is revealed that tangeretin shows not only the inhibitory effects on cell proliferation but also the strong suppressing effects on the ATRA-induced $O_2^-$-generating ability of U937 cells. In addition, polymethoxyflavones show both high membrane permeability and metabolic resistance because all hydroxyl groups in tangeretin are capped by methyl residues (Walle, 2007). Therefore, tangeretin may be expected to be an effective transdermal and oral anti-inflammatory biothrapeutic drug.

## ACKNOWLEDGMENTS

We thank R. Madhyastha for editorial reading of the manuscript. This work was supported in part by JSPS KAKENHI [Grant Number: 18K07804 (to F. K.), 19K02329 (to H. K.) and 22K02173 (to H. K.)] and the Research Center for GLOBAL and LOCAL Infectious Diseases, Oita University [Grant Number: 2022B06 (to H. K.)].

**Conflict of interest----** The authors declare that there is no conflict of interest.

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