CYTOKINE INHIBITORY ACTIVITY OF EURYCOMANONE IN RAW 264.7 CELLS STIMULATED WITH VIRAL-MIMICKING POLY (I:C)

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SUMMARY

Poly (I:C) (Polyinosinic:polycytidylic acid) is a synthetic analog of double-stranded RNA binding toll-like receptor 3 (TLR3) and mimicking a virus infection in macrophage. Here, for the first time, we showed the inhibitory effects of eurycomanone on the production of IL-6, TNF-α and IL-10 of RAW 264.7 cells stimulated with poly (I:C). Eurycomanone inhibited production of pro-inflammatory cytokines including IL-6 and TNF-α of RAW 264.7 cells stimulated with poly (I:C) with the IC$_{50}$ value being 5.14 ± 0.60 μM and 2.32 ± 0.40 μM, respectively. Interestingly, this compound also inhibited the production of anti-inflammatory cytokine IL-10 of RAW 264.7 cells stimulated with poly (I:C) with the IC$_{50}$ value being 14.60 ± 0.32 μM. These results suggested that eurycomanone has a potentially regulatory role on production of both pro-inflammatory and anti-inflammatory cytokines in macrophage in the mimicking context of virus infection. Whether eurycomanone inhibits the binding of poly (I:C)-TLR3 or intracellular signaling pathways needs further investigation.

Keywords: Eurycomanone, poly (I:C), RAW 264.7 cells, inflammatory cytokines

INTRODUCTION

Polyinosinic:polycytidylic acid, poly (I:C), is a synthetic analog of double-stranded RNA mimicking RNA viruses with one strand being a polymer of inosinic acid, one being a polymer of cytidylic acid. This structure has been commonly known to interact with toll-like receptor 3 (TLR3), a single-pass membrane-spanning receptors mainly expressed in the membrane of macrophages (McGarry *et al.*, 2021). Recognition of invasive pathogens like poly (I:C) through TLRs on membrane of immune cells can trigger subsequent activation of downstream signaling cascades and cause expressions of inflammatory mediators like cytokines.

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Depending on certain circumstances, poly(I:C) can selectively activate several signaling pathways after binding to TLR3 that activates the transcription factor interferon regulatory factor 3, which leads to the production of type I IFNs (Fitzgerald *et al.*, 2020). On the other way, recognition of poly (I:C) can lead to the activation of the transcription factor NF-κB, which triggers the production of inflammatory cytokines such as TNF-α, IL-6 and IL-10 (Tanaka *et al.*, 2014; Saraiva *et al.*, 2010). Eurycomanone has been long documented for its aphrodisiac activity, utilizing various pathways, through multiple mechanisms. Interestingly, there are studies that pointed out the relation between the regulation of hormone to that of inflammatory cytokines, suggesting a potential in developing eurycomanone for anti-inflammatory activities (Wu *et al.*, 2018). Recently, our group has found the biological effects of compounds that inhibited the production of pro-inflammatory cytokines in cells stimulated by bacterial-derived LPS, but not yet by viral-mimicked poly (I:C) (Tran *et al.*, 2018; Tran *et al.*, 2022). In addition, eurycomanone has not been thoroughly studied to uncover its anti-inflammatory potentials. In this study, we conducted experiments with the intention of consolidating previous research on the anti-inflammatory properties of eurycomanone, however, in the context of using poly (I:C) as a stimulator mimicking a viral infection.

MATERIALS AND METHODS

Cell culturing and treatment of cells with poly(I:C) and eurycomanone

RAW 264.7 cell line was provided by Dr. T. Kishimoto, Osaka University, Japan. These cells were cultured in RPMI 1640 medium (10% FBS) with 100 μg/mL penicillin, 100 μg/mL streptomycin. Cells were plated into a 90 mm×20 mm petri dish. The culture medium is changed after 1-2 days. Proceed with cell passaging when the cell density reaches 80% confluence. The RAW 264.7 cells were grown in a 24-well plate, containing $5×10^5$ cells/ml. Different concentrations of eurycomanone (1, 3, 10, and 30 μM) were added into the wells for 30 minutes followed by stimulation with 30 μg/mL poly (I:C) (Sigma). Eurycomanone was purchased from ChromaDex Inc. (Irvine, CA). The cells were harvested for qPCR after 6 h and supernatants were harvested for ELISA after 24 h as described previously (Masuda *et al.*, 2011).

Cell viability assay

The MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay was used to determine the viability of RAW 264.7 and HEK293T cells treated with eurycomanone. Cell counting was performed so that the final concentration could be adjusted to $5×10^5$ cells/mL. Each testing well of the 24-well plate was supplemented with 1 mL of cells and then kept incubated in a CO_2 incubator at 37°C, 5% CO_2 for 1 h for complete adhesion. Different concentrations of eurycomanone were added into the wells. On the same test plate, there are control wells with just 10% DMSO solvent, and blank wells with only the medium. The plate was put into the incubator again at 37°C, 5% CO_2 for 24 h. MTT (5 mg/mL) was added to each well about 4 h before adding DMSO. The plate was then placed in a microplate reader to record the absorbance of samples at 540 nm. Cell viability was calculated as described previously (Luz *et al.*, 2022).

$$\% \text{ Cell viability} = \left(\frac{\text{OD}_{\text{treated sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}}\right) \text{X } 100 \text{ (\%)}$$

Real-time quantitative PCR

Total RNA was isolated using RNeasy Mini Kit from Qiagen (Santa Clarita, CA). One mg of total RNA was used to synthesis first stranded cDNA using Maxime RT PreMix. Finally, fluorescence quantitative PCR detection was performed using the PowerUp™ SYBR™ Green Master Mix Kit (ThermoScientific). The primers used for the qRT-PCR amplification are following: β-actin-F 5'-AGCCATGTACGTAGCCATCC-3', β-actin-R 5'-CTCTCAGCTGTGGTGGTGAA-3', IL6-F 5'-GAGGATACCACTCCCAACAGACC-3', IL6-R 5'-AAGTGCATCATCGTTGTTCATACA-3', TNFα-F 5'-AGACCCTCACACTCAGATCATCTTC-3', TNFα-R 5'-CCACTTGGTGGTTTGCTACGA-3', IL10-F 5'-AAGGCAGTGGAGCAGGTGAA-3', IL10-R 5'-CCAGCAGACTCAATACACAC-3' as described previously (Masuda *et al*., 2011).

ELISA

IL-6, IL-10, and TNF-α cytokine levels of the mouse cell line RAW 264.7 were determined by ELISA Flex kit (Mabtech, Abcam) according to the manufacturer's instructions. The optical density was measured in Multiskan FC Microplate Photometer, an ELISA reader, at 450 nm within 15 min after the last step. Data are expressed as the average value of at least 3 repetitions. The IC$_{50}$ value was determined using ImageJ 1.50i computer software (NIH, Maryland).

Statistical analysis

The data are presented as the mean ± standard deviation of three independent experiments. A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Examination of cell viability with eurycomanone treatment

Eurycomanone was tested on RAW 264.7 and HEK293T cells for its cytotoxicity using MTT assay. Cells were measured for their viability after 24 h. The cell viability after various concentrations of eurycomanone treatments showed significantly safer ranges of percentage viabilities in RAW 264.7 cells as 100%, 96%, 98%, 94%, 94% and in HEK 293T cells as 100%, 92%, 83%, 80% (Figure 1).

Eurycomanone exhibited no effect on RAW 264.7 and HEK293T cells. Hence, eurycomanone was proposed to be used for drug testing after further validations.

Evaluation of the anti-inflammatory effect of eurycomanone

The cells were pre-treated with eurycomanone (1, 3, 10, 30 μM) for 30 min before being stimulated with poly (I:C) (30 μg/mL). mRNA expression of three cytokines IL-6, IL-10, and TNF-α was detected by qPCR in 3 h after poly (I:C) stimulation and peaked at 6 h before decreasing at 24 h (data not shown). Then, the effect of eurycomanone on mRNA and protein levels of three cytokines IL-6, IL-10, and TNF-α were determined after 6 h by qPCR (Figure 2) and 24 h by ELISA (Figure

3). For the first time, we showed that this compound at different concentrations was able to inhibit the production of IL-6, IL-10, and TNF-α at both mRNA and protein levels.
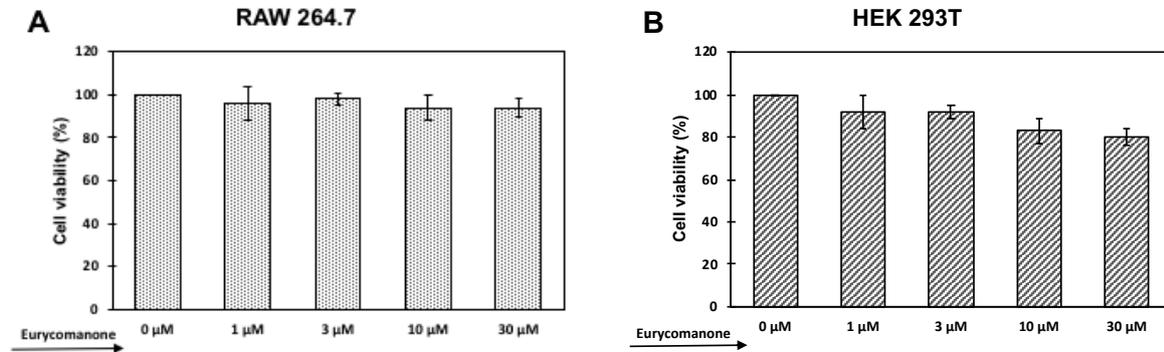
Figure 1. Cytotoxicity of eurycomanone on RAW 264.7 cells (A) and HEK 293T cells (B). The data are presented as the mean ± SD of three independent experiments. Cell viability was calculated was calculated via MTT assay.
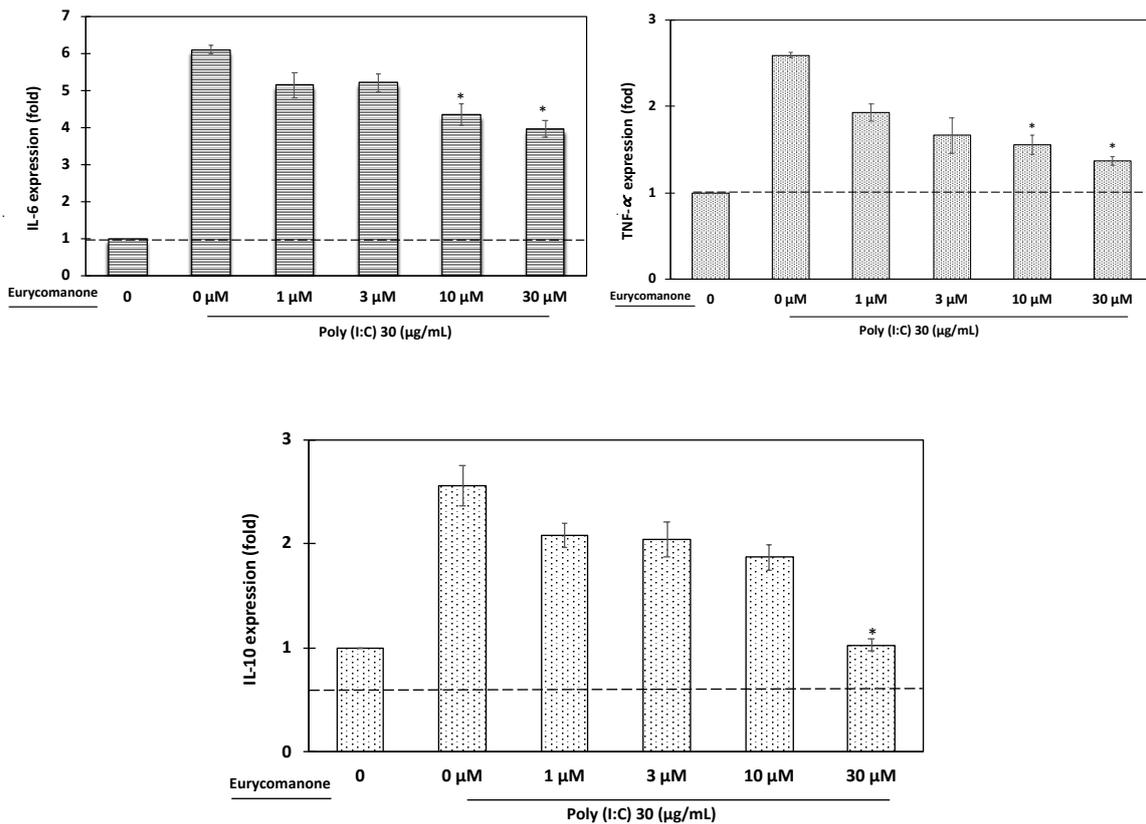
Figure 2. Effects of eurycomanone on the mRNA expression level of *IL-6*, *TNF-α*, and *IL-10* in RAW 264.7 cells in the stimulation of poly (I:C). The data are presented as the mean ± SD of three independent experiments. qPCR was utilized to measure the gene expression level. *$p<0.05$ compared with poly (I:C) only.

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Figure 2 showed that poly (I:C) was able to induce mRNAs of *IL-6*, *TNF-α* and *IL-10* with 6.1, 2.6 and 2.6-fold compare to non-stimulated cells at 6 h, respectively. Eurycomanone inhibited pro-inflammatory cytokine *IL-6* with 15%, 15%, 28% at 1 μM, 3 μM, 10 μM, respectively, and reaching a maximum of 36% at a concentration of 30 μM, compared to untreated cells. Eurycomanone also inhibited pro-inflammatory cytokine *TNF-α* with 24%, 36%, 40% at 1 μM, 3 μM, 10 μM respectively, and reaching a maximum of 48% at a concentration of 30 μM, compared to untreated cells. Interestingly, eurycomanone also inhibited *IL-10*, which commonly considered as an anti-inflammatory cytokine with 20%, 20%, 28% at 1 μM, 3 μM, 10 μM respectively, and reaching a maximum of 60% at a concentration of 30 μM, compared to untreated cells (Figure 2). Then, the production of protein levels of IL-6, TNF-α and IL-10 were examined after 24 h. Figure 3 showed that eurycomanone exerts an IL-6, TNF-α and Il-10 inhibitory activity in poly (I:C)-stimulated RAW264.7 cells with IL-6-IC_{50} values of 5.14 ± 0.60 μM, TNF-α-IC_{50} values of 2.32 ± 0.40 μM and IL-10-IC_{50} values of 14.60 ± 0.32 μM, respectively.
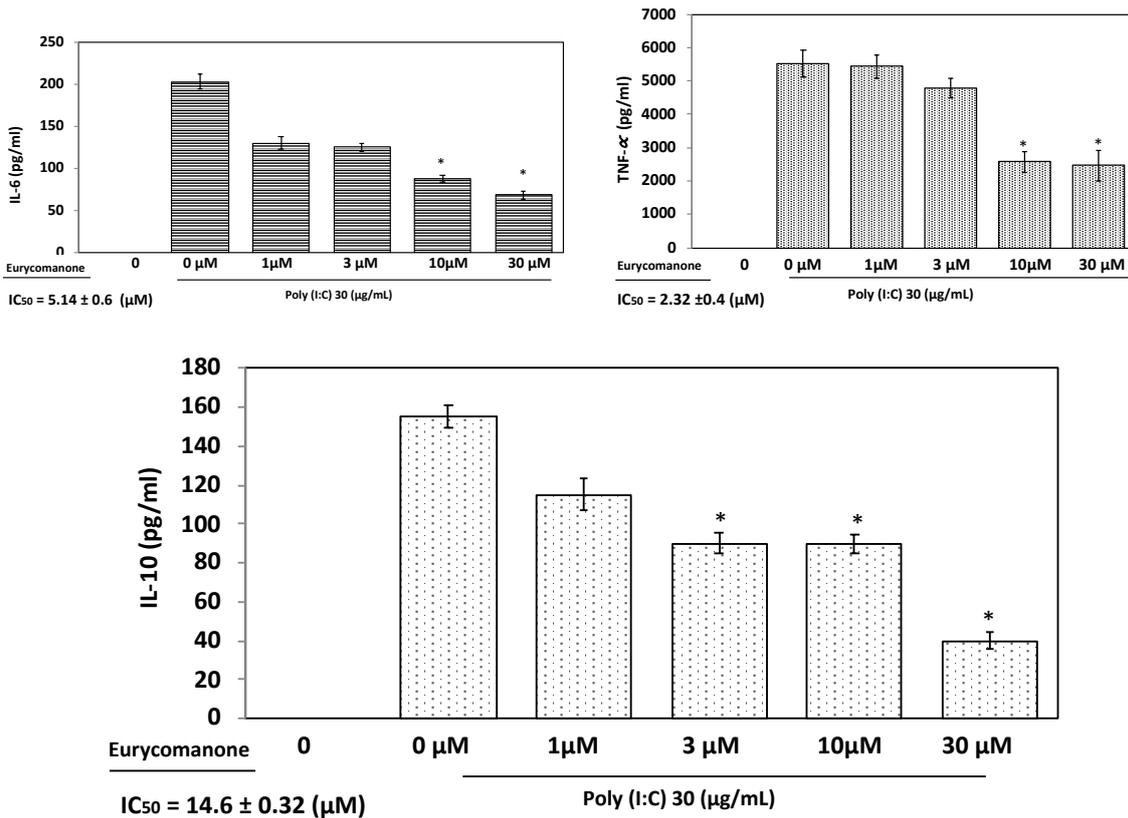
Figure 3. Effect of eurycomanone on IL-6, TNF-*α* and IL-10 in RAW 264.7 cells in the stimulation of poly (I:C). The data are presented as the mean ± SD of three independent experiments. ELISA was utilized to measure the protein level. *$p<0.05$ compared with poly (I:C) only.

These results suggest that eurycomanone is a good anti-inflammatory compound that may develop into a potential drug, typically in the context of virus infection. Mechanistically, it is known that the production of pro-inflammatory cytokines in activated macrophages is regulated by various intracellular pathways such as the NF-κB pathway (Tanaka *et al*., 2014). Eurycomanone has been shown to significantly suppressed NF-κB reporter activity in cells that could be secondary to explaining the results of the compound on their anti-inflammatory effect (Hajjouli *et al*., 2014). Eurycomanone has been long studied for its ability to significantly increase the testosterone and decrease the estrogenic level (Low *et al*., 2013). Testosterone has demonstrated its immunosuppressive role by decreasing pro-inflammatory cytokine expression like IL-6, TNF-α and IL-10 via a variety of pathways and mechanisms that can be occurred through the inhibition of NRF1-derived NF-κB signaling pathway (Liva *et al*., 2001; Fijak *et al*., 2011; Wang *et al*., 2021). Additionally, estrogen proved to induce IL-10 production in multiple settings, for example, though exerting negative effects on expression of pro- and anti-inflammatory mediator genes (Maciuszek *et al*., 2020). We thus hypothesized that the regulatory effect of eurycomanone at specific concentrations on cytokine production under poly (I:C) stimulation in RAW cells could be linked with the balance of testosterone/estrogen. Recently, we reported that several plant-derived compounds showed anti-inflammatory effects in LPS-TLR4 signaling by activating Ahr signalling pathway (Tran *et al.,* 2022). Interestingly, Ahr signaling also is able to regulate sex hormones (Ohtake *et al.,* 2008). Whether eurycomanone interacts between Ahr-sex hormone signaling in the

macrophages stimulated with poly (I:C)-TLR3 that may inhibit inflammatory cytokine production needs further examination.

CONCLUSION

Our study for the first time provides the evidence on anti-inflammatory activity of eurycomanone in RAW 264.7 cells under the stimulation of viral-mimicking poly (I:C). The cytokines including pro-and anti-inflammatory cytokines such as IL-6, TNF-α and IL-10 were inhibited with different concentrations of eurycomanone. Our study suggests a potential therapy using eurycomanone in anti-viral immune responses.

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