

Supplementary Material-2

Cell culture and *in vitro* evaluation of the anti-inflammatory effect of RE

Cell culture

Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin and 10% fetal bovine serum. Cells were incubated in a humidified environment with 5% CO₂ at 37°C. Cells at passages 10-15 were used for experiments.

Cytotoxicity analysis of RE

To choose the safe concentration of RE, RAW 264.7 cells were seeded in a 96-well plate at density 2×10^4 cells and stabilized for 24 h. The media was removed and cells were then treated with different concentrations of RE (solubilized in DMSO) in the presence and/or absence of LPS (100 ng/mL) for next 24 h. After the supernatants were removed, the cells were incubated with MTT solution (5 mg/mL of RPMI media) for 4 h in a humidified atmosphere of 5% CO₂ at 37°C. The medium was then aspirated from each well and was dissolved in dimethyl sulfoxide (100 µL). The absorbance at 570 nm was determined using a microplate reader (BioTek, Synergy 4, USA) and the cell viability of the control cells (only DMSO) was set at 100%.

***In vitro* evaluation of the anti-inflammatory effect of RE**

RAW 264.7 cells were plated at 1×10^5 cells/well in 24-well plate for 24 h. After 24 h, the cells were pretreated with RE at two different concentrations, 5 and 10 µg/ml (selected based on cytotoxicity of RE) for 1 h and then stimulated with 100 ng/mL of LPS for next 18 h at 37°C in a humidified atmosphere of 5% CO₂. After 18 h of stimulation, the culture supernatant was collected and centrifuged (4000 rpm for 10 min) to remove cellular debris.

The amount of secreted TNF- α and IL-6 in the supernatants was determined using respective ELISA kits (BD Bioscience, San Diego, CA, USA). The concentration of nitrite as an indicator of nitric oxide production was determined by using the Griess reagents (Sigma-Aldrich Co, St Louis, MO, USA) and sodium nitrite as standard.

Western blot analyses were performed using RAW 264.6 cell lysates to assess the effects of RE on the expressions various inflammation related proteins including NF- κ B (p65), I κ B α , p-I κ B α , iNOS and COX-2. The RAW 264.6 cells were plated at 2×10^6 cells/well in 6-well plate and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂. After 24 h, the cells were pretreated with RE at two different concentrations, 5 and 10 μ g/ml for 1 h and then stimulated with 100 ng/mL of LPS for next 18 h. Cultures were rinsed twice with ice-cold PBS and then triturated in RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA) supplemented with 1% Halt protease inhibitor cocktail to obtain whole protein extracts. Similarly, nuclear extracts from cell pellets were obtained by using NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA). The concentration of protein in whole and nuclear extracts was determined by Bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA) and an equal amount of protein (30 μ g/lane) lysate were subjected to western blot analysis and the quantification of iNOS, COX-2, I κ B α , p-I κ B α and NF- κ B (p65) expressions as described below.