Materials and methods

Preparation of green tea tree petiole extract

Green tea tree petiole extract was prepared with following procedures briefly. The green tea tree, *Camellia sinensis,* petioles were collected from the Jangwon Green Tea Tree Garden (Jeju, Republic of Korea). The voucher specimen (DRSD-1312-008) was deposited at Durae corporation, Korea. Dried and ground green tea tree petioles (100g) were extracted with 1L water for 6h at 90℃. The aqueous solution was obtained after centrifuge and concentrated to make the volume to 100ml. 40ml of 95% ethanol was added to the aqueous solution and centrifuged to remove the insoluble part by centrifuge. Chlorophylls were removed by adding 240ml of 95% ethanol and it was repeated once more. The final extract was obtained by filtering with 3μm filter paper and evaporating the ethanol. The final yield was 1g of green tea tree petiole powder.

HPLC analysis of green tea tree petiole extract

Before HPLC analysis, green tea tree petiole extract was processed for protein digestions. Extract was incubated with Bromelanin of pH 6.0-7.0 for overnight at 50℃. Then enzyme was inactivated by heating of 85℃ for 30 mins. Peptides sample was prepared by freeze dry after filtering. Borate buffer and Flour reagent 2A from AccQ-Tag amino acids derivatization kit were used for amino acids analysis. HPLC analysis of GTP amino acids was carried out with ACQUITY UPLC AccQ-Tag Ultra C18 Column (2.1 X 100 mm, 1.7 μm). AccQ-Tag Ultra Eluent A (buffer) and B (1% formic acid in Acetonitrile) were taken as the mobile phase. With the tea extract, chromatographic analysis for standard chemical such as catechins was carried out. The concentration of the chemical in the tea extract was calculated from the calibration curve of the standard chemical integral area.

Cell culture

Human neonatal epidermal keratinocytes (HEKs) were cultured in KGM-Gold™ medium including KGM-Gold growth Bulletkit from Lonza (Basel, Switzerland). HEKs were used at passage number 3 ~ 7 in the experiments and serially passaged at 70–80% confluence. Cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

Cell viability assay

Cells were seeded onto 96-well plates (1×10^4 cells/well) and cultured with GTP extract for 24 h, after which an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution was added and cells were incubated for 30 min. The dark blue formazan crystals formed in intact cells were dissolved in dimethylsulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader.

3D human skin equivalent

EFT (EpiDerm Full-Thickness)-400 containing normal human dermal fibroblasts and epidermal keratinocytes were purchased from MatTek Corporation (Ashland, MA, USA). The EpiDerm skins were maintained in the EFT-400-ASY medium as recommended by the manufacturer. First, the EpiDerm skins were incubated for 3 days under 5% CO_2 condition. And then, 10μg/ml poly(I:C) was added to EpiDerm culture medium on day 4, 6, and 8 and 50ppm GTP extract was 30min pre-treated topically to EpiDerm on day 4, 6, 8 and 11. PBS was taken as a negative control. Tissues were frozen on day 13 for histological analysis.

RNA extraction and Real time- polymerase chain reaction (PCR)

Total RNA from human epidermal keratinocytes was extracted using Trizol (Life Technologies, Grand Island, NY, USA) and cDNA was produced by SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). RT-qPCR was performed with TaqMan assay (Applied Biosystems, Foster city, CA, USA). The primer sets for qRT-PCR in the Taq-Man® Gene Expression Assay were purchased from Applied Biosystems. Target gene expression was normalized against expression of the housekeeping gene, GAPDH.

Immunostaining

Immunostaining was performed based on the previous study.[17] Unfixed 3D human skin equivalent embedded in Optimal Cutting Temperature compound (Tissue-Tek® O.C.T™, Sakura, USA) was frozen in liquid nitrogen, sectioned at 6 ㎛ thick using cryostat (CM1950, Leica, Germany), and stained with hematoxylin and eosin (H&E). Replicate cryosection was immunohistochemically stained using polyclonal rabbit anti-MMP-1 antibody (ab38929, Abcam, Cambridge, MA, USA) as the primary antibody and anti-rabbit HRP conjugated antibody (ab6802, Abcam, Cambridge, MA, USA) as the secondary antibody. Immunoreactivity was visualized using 3,3'-Diaminobenzidine as a chromogen. The result was analyzed under light microscope (BX53, Olympus, Japan) and photomicrographs were taken using cooling digital camera (DP72, Olympus, Japan).

Enzyme-linked immunosorbent assay (ELISA) for MMP-1

MMP-1 ELISA kit from R&D systems was used for determination of the expression level of MMP-1. The cell culture supernatant from HEKs that were incubated with GTP extract for 2 days together with poly(I:C) was taken for ELISA. ELISA was performed according to the manufacturer's protocol.

Statistical analysis

Statistical analysis of the data was performed using one-way ANOVA and the paired Student's t-test. Results are presented as means ± SD. Differences with a $p<0.05$ were considered statistically significant.