ABSTRACT: Prostaglandin (PG) E2 is an important mediator of skin wound healing without excessive scarring and gastric ulcer healing. However, PGE2 has a short lifetime in vivo because it is metabolized rapidly by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). Ethanol extract of Eriobotryae folium (EFEE) elevated intracellular and extracellular PGE2 levels in HaCaT cells and inhibited 15-PGDH (ED50: 168.4 μg/mL) with relatively low cytotoxicity (IC50: 250.0 μg/mL). Real-time PCR analysis showed that mRNA expression of cyclooxygenase (COX)-1 and COX-2 enzymes were increased and prostaglandin transporter (PGT) was decreased in HaCaT cells by EFEE. Moreover, wound healing effect of EFEE (168.4 μg/mL) was comparable to that of TGF-β1 (300 pg/mL) as a positive control. These results demonstrate that EFEE may be valuable therapeutic materials for the treatment of PGE2 level dependent diseases.

Key Words: Eriobotryae Folium, Prostaglandin E2, Wound Healing

INTRODUCTION

Eriobotrya japonica (Thunb.) Lindley is an evergreen tree of the Rosaceae family. The plant originated in southeastern China and later became naturalized in Japan, India, Korea and many other countries. The dried leaf of E. japonica (Eriobotryae folium) is a famous traditional medicine for treatment to clear away lung-heat, eliminate phlegm, lower the adverse-rising energy, relieve cough and regulate the stomach to restrain vomiting (Ito et al., 2000). Especially, various triterpenes, sesquiterpenes, flavonoids and tannins have been found in the Eriobotryae folium. Moreover extract and isolated compound from leaves of this plant have been reported to be physiologically active, exhibiting antioxidant, anti-inflammatory, antitumor, hypoglycemic effect, liver protective effect and antimutagenic (Banno et al., 2005; Bae et al., 2010; Cha et al., 2011a, 2011b; Choi et al., 2011). Moreover, antifibrosis effects of triterpene acids of Eriobotryae folium has been reported (Yang et al., 2012).

Prostaglandins are a family of biologically potent fatty acids derived from arachidonic acid (AA) through the cyclooxygenase (COX) pathway. Prostaglandin (PG) E2 acts as both an inflammatory mediator and fibroblast modulator (Sandulache et al., 2002). In addition, COX is a rate-limiting enzyme in the biosynthesis of PGs from AA, and exists in two isoforms (COX-1 and COX-2). PGs are not stored in cells but are released into the cellular environment, where they exert autocrine or paracrine effect on neighboring cells. Synthesized PGE2 is simply diffused
and actively extruded by the multidrug resistance-associated protein 4 (MRP4) from the cells (Schuster, 2008). After acting via its PGE$_2$ receptor (EPR), pericellular PGE$_2$ is cleared via re-uptake by PG transporter (PGT) and then rapidly metabolized by cytosolic enzyme named NAD$^+$-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Schuster, 1998). PGE$_2$ has been identified as an important mediator for gastric ulcer healing (Chatterjee et al., 2012; Yamamoto et al., 2012; de-Faria et al., 2012), dermal wound healing (Choi et al., 2013; Wilgus et al., 2004) and anti-fibrotic activity (Zhou et al., 2012).

In this study, the ethanol extract of Eriobotryae folium (EFEE) as a 15-PGDH inhibitor after screening plant extracts was selected and investigated the regulation and expression of COX-1, COX-2, MRP4 and PGT in fibroblast cell line (HaCaT) for the regulation of PGE$_2$ level. Moreover, the effect of EFEE during wound healing in HaCaT cells was investigated.

**MATERIALS AND METHODS**

1. **Plant materials and extraction**
   Eriobotryae folium was collected from Wando, Jeonnam, Korea in June 2013. It was authenticated by Professor BS Pyo of the department of Oriental Medicine Materials, Dongshin University and the voucher specimen (No. DSUBIC-13-02) was preserved in the herbarium of the Biotechnology Industrialization Center of Dongshin University. Plant material was shade dried at room temperature, chopped followed by pulverization. Two hundred grams of powdered plant material were extracted by reflux with 94.0% ethanol for 3 h. The extract was evaporated to dryness by using a rotary vacuum evaporator at 50$^\circ$C to get crude extract (EFEE, 38 g).

2. **Instruments and reagents**
   PGE$_2$, NAD$^+$, NADH, glutathione-sepharose 4B, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), EDTA, reduced glutathione, and rest of essential chemicals and reagents were purchased from Sigma (St. Louis, MO, USA). The GST gene fusion pGEX-2T expression vector was purchased from Pharmacia Crop. (Piscataway, New Jersey, USA). The cDNA of human 15-PGDH was cloned from a human placenta cDNA library, as described previously (Ensor et al., 1990). The UV spectra were obtained using a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). PGE$_2$ enzyme immunoassay kit was purchased from Thermo Scientific (Rockford, IL, USA). Real-time PCR was performed with the Light Cycler 2.0 Instrument (Roche, Mannheim, Germany).

3. **Expression and purification of 15-PGDH**
   15-PGDH cDNA plasmid containing BamHI and EcoRI sites of the pGEX-2T expression vector was used to transform Escherichia coli BL-21 lysS. The cells were grown in 500 mL medium containing 50 $\mu$g/mL ampicillin at 37$^\circ$C and 220 rpm until the OD$_{600}$ reached 0.6. Isopropyl B-D-thiogalactoside (1 M stock solution) of 500 $\mu$L was added and the cells were allowed to grow for 12 h at 25$^\circ$C. Then the cells were harvested by centrifugation at 4000 $\times$ g for 30 min at 4$^\circ$C. The cell pellets were resuspended in 20 mL cold cell Lysis buffer (1×PBS buffer pH 7.4 containing 1 mM EDTA and 0.1 mM DTT) and sonicated (4×10 s at 4$^\circ$C). The disrupted cells were centrifuged at 4000 $\times$ g for 20 min at 4$^\circ$C. The supernatant was applied slowly to a glutathione-sepharose 4B column, which was equilibrated at 4$^\circ$C with a lysis buffer. The 15-PGDH was eluted from the glutathione-sepharose 4B column by incubation at room temperature for 5 min with the elution buffer (50 mM Tris-HCl pH 8.0 containing 10 mM reduced glutathione, 1 mM EDTA and 0.1 mM DTT). The concentration of enzyme was determined and the purity of the 15-PGDH was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

4. **15-PGDH assay**
   Assay for the activity of the 15-PGDH inhibitors was performed using a fluorescence spectrophotometer by measuring the formation of NADH at 468 nm following excitation at 340 nm. Tris-HCl buffer (50 mM, pH 7.5) containing 0.1 mM DTT, 0.25 mM NAD$^+$, purified enzyme (10 $\mu$g), 21 $\mu$M PGE$_2$ and various concentrations of EFEE (total 2 mL) were added to each cell. Each concentration was assayed in triplicate. The absorbance at 340 nm after incubation with EFEE was determined from a standard curve of various concentrations of NADH.

5. **Cell culture and cell viability assay**
   HaCaT cells, a human keratinocyte cell line, were
cultured in Dulbecco’s modified Eagle’s media (DMEM). The cultured media were supplemented with 10% heat inactivated fetal bovine serum (Sigma, St. Louis, MO, USA) and 100 μg/mL penicillin, in 5% CO₂ at 37°C. Cell viability was determined by the MTT assay. HaCaT cells (1 × 10⁵) were seeded in 96 well plates per 90 μL of DMEM medium. After the overnight of incubation, EFEE was treated for 72 h followed by 4 h of incubation with 10 μL of MTT (5 μg/mL stock solution). Then medium was removed and followed by addition of 150 μL of DMSO to dissolve formazan. Absorbance was measured at 540 nm using an microplate reader (Perkin-Elmer, Waltham, MA, USA).

6. Determination of PGE₂ release

HaCaT cells was seeded (5 × 10⁵ cells/well) on to 6-well culture plates in DMEM medium containing fetal bovine serum and antibiotic for overnight in 5% CO₂ incubator at 37°C. EFEE of different concentrations were treated and media was collected after 12 h of sample treatment. PGE₂ levels were determined by PGE₂ enzyme immunoassay kits according to manufacturer’s protocol.

7. Quantitative real-time PCR

Total cellular RNA was isolated from HaCaT cells using TRI reagent (RNAiso Plus, Takara Bio Inc., Shiga, Japan) according to manufacturer’s protocol. cDNA for each RNA sample was synthesized in 20 μL reactions using the SuperScript First Strand synthesis system for reverse transcription-PCR (Invitrogen, Carlsbad, CA, USA) following manufacturer’s protocol. PCR reaction contained 4 μL of 1:5 diluted cDNA, 4 mM MgCl₂, 10 pmole of each primer and 4 μL of Fast Starter Mix buffer (dNTPs, SYBR Green dye and Tag polymerase). Primers used for real-time PCR were as follows: Human PGT forward, 5′-GGATGCTTGGAGGAATCCCTCA-3′ and reverse, 5′-GCAC GATCCTGTCTTTGCTGAA-3′; MRP4 forward, 5′-AACCTCTAACC GACATTCCTG-3′ and reverse, 5′-TCAATGAGGGCCACCATC-3′; COX-1 forward 5′-CCTCATGTTTGCCTTCTTG-3′ and reverse, 5′-GGCGGGTACATTCTCCATC-3′; COX-2 forward, 5′-GATCTACCCTCCTCA-3′ and reverse 5′-GAACAACTGCTCATCAC-3′ and β-actin forward 5′-GACTATGACTTAGTTGCGTTA-3′ and reverse 5′-GTTGAAC TCTCTACATACTTCCG-3′.

8. Wound healing effect by in vitro scratch assay

For the in vitro scratch assay (Hintermann et al., 2001; Koivisto et al., 2006), HaCaT cells were seeded onto six well plates in a density of 5 × 10⁵ cells/well, and grown until they reached about 80% confluence. Then the media was changed with serum free DMEM containing mitomycin (10 μg/mL) and incubated for 2 h to prevent wound proliferation followed by extensive washing with PBS. A scratch was made using a sterile 200 μL pipette tip and cells were washed. TGF-β1 (300 pg/mL) as a positive control and EFEE as a 15-PGDH inhibitor were added to the medium. Pictures were taken exactly at the same position before and after the incubation to document the wound healing process. Experiments were repeated twice and delegate pictures are shown. Scratches were picture under the microscope (×100) immediately after scratch making and once more after 48 h incubation at 37°C, 5% CO₂ incubator.

9. Statistical analysis

The results are expressed as the mean ± SD. Data between groups were analyzed by a Student’s unpaired two-tailed t-test and p-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

1. Determination of extracellular and intracellular PGE₂ levels

PGE₂ has been known as an important mediator of healing of gastric ulcer, dermal wound and so on (Chatterjee et al., 2012; Yamamoto et al., 2012; Choi et al., 2013; Li et al., 2011). It is reported that 15-PGDH is inhibited by a variety of pharmacological agents including NSAIDs such as indomethacin, anti-paltelet aggregatory drugs such as panaxynol (Moustafa et al., 2013; Fujimoto et al., 1998), anti-allergic drugs such as flavonoid baicalein (Iijima et al., 1980), and so on. As shown in Table 2, EFEE increased 18.33% of extracellular PGE₂ level and 39.68% of intracellular PGE₂ level compared to control group in HaCaT cells.

2. 15-PGDH inhibitory activity and cytotoxicity

15-PGDH is the key metabolic senzyme of PGE₂ (Choi et al., 2013). Therefore, inhibition of 15-PGDH is supposed
to facilitate previous biological effects by increasing PGE$_2$. Inhibitors of 15-PGDH will be valuable for the disease therapy requiring PGE$_2$ elevation (Moustafa et al., 2013).

For this reason, we have searched EFEE for potential 15-PGDH inhibitory activity. The effective dose for 50% inhibition (ED$_{50}$) was used for the expression of 15-PGDH inhibitory activity. We found that EFEE contained 15-PGDH inhibitor ED$_{50}$ value, 168.4 µg/mL (Table 1). On the other hand, cytotoxicity is very important for pharmaceutical application. The cytotoxicity of EFEE was determined by MTT assay. IC$_{50}$ (concentration for 50% of cell survival) of EFEE in HaCaT cells appeared was 250.0 µg/mL.

3. COX-1, COX-2, MRP4 and PGT expression

COX pathway increased PGE$_2$ level by inducing the synthesis of PGE$_2$ from arachidonic acid in biological system. In this connection, expression of COX-1, COX-2, MRP4 and PGT is essential for PGE$_2$ synthesis and transportation. PGE$_2$ can cross through the membrane by simple diffusion or via a prostaglandin efflux transporter, such as MRP4. After acting via its EPR, pericellular PGE$_2$ is cleared via re-uptake by PGT and then rapidly metabolized by cytosolic 15-PGDH (Lee et al., 2012). Thus, PGE$_2$ levels would be functions of expression levels of these genes. Therefore, we treated HaCaT cells with EFEE and checked the regulation of COX-1, COX-2, MRP4 and PGT. Real-time PCR assay showed that EFEE increased expression of COX-1 and COX-2 (Fig. 1). On the other hand, expression of MRP4 and PGT decreased by EFEE treatment. In Table 2 and Fig. 1, intracellular and extracellular PGE$_2$ level in HaCaT cells elevated by the increasing of COX-1 and COX-2 expression, and the decreasing of MRP4 and PGT expression. In addition, EFEE contained 15-PGDH inhibitory effect (Table 1). Interestingly, it has been reported that Eriobotryae folium extract suppressed LPS-induced nitric oxide and PGE$_2$ production through the inhibition of inducible nitric oxide synthase and COX-2 expression in lipopolysaccharide-stimulated RAW264 cells (Uto et al., 2010). Therefore, it is estimated that EFEE has different functions working in different cells such as murine macrophages, keratinocytes.

4. Wound healing effect by in vitro scratch assay

For the wound healing study, in vitro scratch assay was performed to compare the result of samples. EFEE
facilitated wound healing as compared to the negative control. Moreover, the wound healing effect of EFEE (168.4 µg/mL) was comparable to that of TGF-β1 (300 pg/mL) as a positive control. TGF-β1 is one of endogenous growth factors, including EGF, FGF and PDGF, that are released from some T cells at the around site and presumed to be a necessary part of wound healing (Liu et al., 2006; Strutz et al., 2001). By measuring the distance of scratch, the recovery % of samples compared with negative control was calculated. EFEE treatment group showed 228% recovery, while TGF-β1 treatment group showed 241% recovery of scratched wounds (Fig. 2).

Many studies have reported that PGE₂ is an important mediator of dermal wound healing (Kolodsick et al., 2003; Savla et al., 2001). Likewise, there is a close connection between wound healing effect of EFEE and PGE₂ up-regulation by controlled expression of MRP4, PGT, COX-1 and COX-2. Moreover, 15-PGDH inhibition effect of EFEE facilitated PGE₂ up-regulation. These results indicate that EFEE could be applicable to therapeutic materials for the treatment of PGE₂ level dependent diseases such as dermal wound, gastric ulcer and so on.

REFERENCES


Fig. 2. Pictures (A) of wound healing effect (B) in HaCaT cells by in vitro scratch assay. EFEE; ethanol extract of Eriobotryae folium. HaCaT cells were treated with EFEE (168.4 µg/mL) and TGF-β1 (300 pg/mL) as a positive control. The pictures were taken at 0 h and 48 h after treatment of samples. Values are mean ± SD (n = 3). **p < 0.001 compared to the control group.


