韓藥作誌(Korean J. Medicinal Crop Sci.) 22(6) : 457 – 462 (2014)
ISSN(Print) 1225-9306
ISSN(Online) 2288-0186
http://www.medicinalcrop.org
http://dx.doi.org/10.7783/KJMCS.2014.22.6.457

피부 각질세포에 대한 비파엽 에탄올 추출물의 PGE_2 조절 작용과 상처치료 효과

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Prostaglandin E_2 Up-regulation and Wound Healing Effect of the Ethanol Extract of Eriobotryae Folium in Human Keratinocyte

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ABSTRACT : Prostaglandin (PG) E_2 is an important mediator of skin wound healing without excessive scarring and gastric ulcer healing. However, PGE_2 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 PGE_2 levels in HaCaT cells and inhibited 15-PGDH (ED_{50} : 168.4 μg/mL) with relatively low cytotoxicity (IC_{50} : 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 PGE_2 level dependent diseases.

Key Words **:** Eriobotryae Folium, Prostaglandin E_2, 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 physiological 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) E_2 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 PGE_2 is simply diffused

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Received 2014 September 22 / 1st Revised 2014 October 7 / 2nd Revised 2014 November 3 / 3rd Revised 2014 November 6 / Accepted 2014 November 16

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) (Schuste, 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℃ to get crude extract (EFEE, 38 g).

2. Instruments and reagent

PGE_2, NAD^+, NADH, gluthathione-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 ㎍/mL ampicillin at 37℃ and 220 rpm until the OD_{600} reached 0.6. Isopropyl B-D-thiogalactoside (1 M stock solution) of 500 μL was added and the cells were allowed to grow for 12 h at 25℃. Then the cells were harvested by centrifugation at 4000 × g for 30 min at 4℃. 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℃). The disrupted cells were centrifuged at 4000 × g for 20 min at 4℃. The supernatant was applied slowly to a gluththione-sepharose 4B column, which was equilibrated at 4℃ with a lysis buffer. The column was washed with lysis buffer until OD_{280} reached below 0.005. 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 ㎚ following excitation at 340 ㎚. Tris-HCl buffer (50 mM, pH 7.5) containing 0.1 mM DTT, 0.25 mM NAD^+, purified enzyme (10 ㎍), 21 μM 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 ㎚ 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 ㎍/mL penicillin, in 5% CO_2 at 37℃. Cell viability was determined by the MTT assay. HaCaT cells (1×10^4) 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 ㎎/mL stock solution). Then medium was removed and followed by addition of 150 μL of DMSO to dissolve formazan. Absorbance was measured at 540 ㎚ using an microplate reader (Perkin-Elmer, Waltham, MA, USA).

6. Determination of PGE_2 release

HaCaT cells was seeded (5×10^5 cells/well) on to 6-well culture plates in DMEM medium containing fetal bovine serum and antibiotic for overnight in 5% CO_2 incubator at 37℃. EFEE of different concentrations were treated and media was collected after 12 h of sample treatment. PGE_2 levels were determined by PGE_2 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 $MgCl_2$ 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'-GGATGCTGTTTGGAGGAATCCTCA-3' and reverse, 5'-GCAC GATCCTGTCTTTGCTGAA-3'; MRP4 forward, 5'-AACCTCTAACC GACATTCCTG-3' and reverse, 5'-TCAA CATATTACAGCCACCATC-3'; COX-1 forward 5'-CCTCAT GTTTGCCTTCTTTGC-3' and reverse 5'-GGCGGGTACATT TCTCCATC-3'; COX-2 forward, 5'-GATCTACCCTCCTCA A-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 5×10^5 cells/well, and grown until they reached about 80% confluence. Then the media was changed with serum free DMEM containing mitomycin (10 ㎍/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℃, 5% CO_2 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 PGE_2 levels

PGE_2 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 PGE_2 level and 39.68% of intracellular PGE_2 level compared to control group in HaCaT cells.

2. 15-PGDH inhibitory activity and cytotoxicity

15-PGDH is the key metabolic senzyme of PGE_2 (Choi *et al*., 2013). Therefore, inhibition of 15-PGDH is supposed

Table 1. 15-PGDH inhibitory activity and cytotoxicity of ethanol extract of Eriobotryae folium (EFEE) in HaCaT cells.

	Concentrations
15-PGDH inhibition (ED_{50})	168.4 ㎍/mL
Cytotoxicity (IC_{50})	250.0 ㎍/mL

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 ㎍/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 ㎍/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

Table 2. Intracellular and extracellular PGE_2 levels after the treatment of ethanol extract of Eriobotryae folium (EFEE) in HaCaT cells.

	Intracellular (pg/㎍)	Extracellular (pg/mL)
Control	1.89 ± 0.20	393.67 ± 12.46*
EFEE	2.64 ± 0.50*	465.84 ± 47.42

Values are mean ± SD (n = 4). *$p < 0.05$ compared to the control group.

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

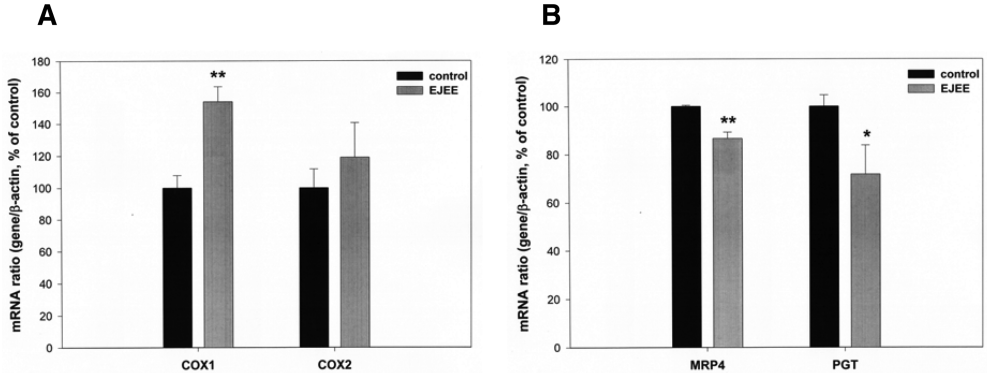

Fig. 1. Effect of ethanol extract of Eriobotryae folium (EFEE) on mRNA expression of COX-1, COX-2 (A) and MRP4, PGT (B). HaCaT cells were treated with EFEE (168.4 ㎍/mL) for 12 h. Values are mean ± SD (n = 4). *$p < 0.05$ and **$p < 0.001$ compared to the control group.

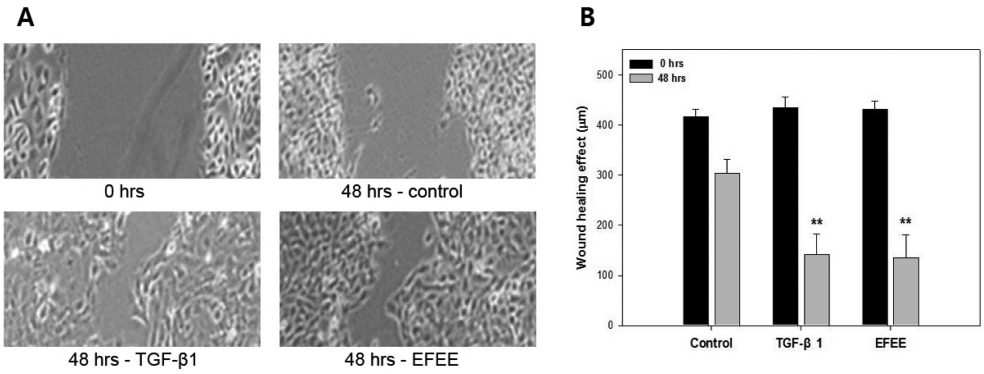

Fig. 2. Pictures (A) of wound healing effect (B) in HaCaT cells by *in vitro* scratch assay. EFEE; ethanol extract of Eriobotryae folium. HaCaT cells were treated with EFEE (168.4 ㎍/mL) and TGF-β1 (300 pg/mL) as a positive control. The pictures were taken at 0 h and 48 h after treatment of samples. Values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

facilitated wound healing as compared to the negative control. Moreover, the wound healing effect of EFEE (168.4 ㎍/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 PGE_2 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 PGE_2 up-regulation by controlled expression of MRP4, PGT, COX-1 and COX-2. Moreover, 15-PGDH inhibition effect of EFEE facilitated PGE_2 up-regulation. These results indicate that EFEE could be applicable to therapeutic materials for the treatment of PGE_2 level dependent diseases such as dermal wound, gastric ulcer and so on.

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