Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

In-vitro Wound Healing Effect of 15-Hydroxyprostaglandin Dehydrogenase Inhibitor from Plant

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Submitted: 30-07-2016 Revised: 08-09-2016 Published: 07-04-2017

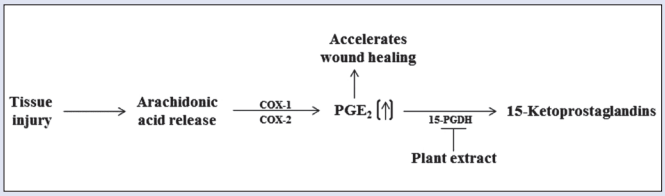
ABSTRACT

Background: Prostaglandins (PGs) have short existence *in vivo* because they are rapidly metabolized by NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) to 15-ketoprostaglandins. Inhibition of 15-PGDH causes elevated level of PGE_2 in cellular system. It will be valuable for the therapeutic management of diseases requiring elevated PGE_2 levels, like wound healing. **Objective:** Ninety-eight plant samples were screened for the discovery of potent 15-PGDH inhibitor. Among them, top five plant extracts as potent 15-PGDH inhibitor were chosen to determine PGE_2 release from HaCaT (Keratinocyte cell line) cell line. Finally, top 15-PGDH inhibitor was selected to evaluate *in vitro* wound healing effect on HaCaT scratch model. **Method:** The inhibitory activity for 15-PGDH inhibitors was evaluated using fluorescence spectrophotometer by measuring the formation of NADH at 468 nm following excitation at 340 nm. Cell viability assay and PGE_2 release was evaluated in HaCaT cell line after treatment of 15-PGDH inhibitors. Scratches were made using sterile 200 µL on HaCaT cell and wound-healing effect was evaluated after treatment of 15-PGDH inhibitor. **Results:** 15-PGDH inhibitors elevated PGE_2 levels in concentration-dependent manner. Ethanol extract of *Artocarpus heterophyllus* (EEAH), the most potent 15-PGDH inhibitor (IC_{50} = 0.62 µg/mL) with least cytotoxicity (IC_{50} = 670 µg/ml), elevated both intracellular and extracellular PGE_2 levels. EEAH facilitated *in-vitro* wound healing in a HaCaT (Keratinocyte cell line) scratch model. **Conclusion:** EEAH might apply to treat dermal wounds by elevating PGE_2 levels via COX-1 induction and 15-PGDH inhibition.

Key words: Prostaglandins, cyclooxygenase, PGE_2, wound healing, 15-hydroxyprostaglandin dehydrogenase

SUMMARY

- Biological inactivation of 15-PGDH causes elevated level of PGE_2 which will be useful for the management of disease that requires elevated level of PGE_2.



Abbreviations used: 15-PGDH: 15-hydroxyprostaglandin dehydrogenase, COX: Cyclooxygenase, DTT: Dithiothreitol, DMEM: Dulbecco's modified Eagle's media, EEAH: Ethanol extract of Artocarpus heterophyllus, MRP4: Multidrug resistance 4, PGs: Prostaglandins, PGT: Prostaglandin transporter, SDS: Sodium dodecylsulfate

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DOI: 10.4103/0973-1296.203971

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INTRODUCTION

Prostaglandins (PGs) are lipid compounds that participate in a variety of physiologic and pathologic processes, and among them, PGE_2 is a major mediator for inflammation.[1] PGE_2 is formed by PG synthetase from PGH_2 through the cyclooxygenase (COX) pathway. Two types of COX have been identified: (i) COX-1 has been expressed constitutively in various tissues, including stomach, and (ii) COX-2 has been induced by cytokines, growth factors, tumor promoters, and other agents.[2] Newly synthesized PGE_2 simply diffused and actively extruded by the multidrug resistance 4 (MRP4) from the cells.[3] Subsequently, EPR receptor is activated followed by pericellular PGE_2 is cleared via re-uptake of PGE_2 by PG transporter (PGT)[4] and then rapidly metabolized by cytosolic enzyme named NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH).[5] This enzyme is expressed ubiquitously in mammalian tissues and responsible for biologic inactivation of PGE_2 to 15-ketoprostaglandins.[6]

PGE_2 has been known as an important mediator for bone formation,[7,8] gastric ulcer healing,[9,10] and dermal wound healing.[11,12] Additionally, PGE_2 has been used to treat gastric ulcer in spite of high price and low efficacy.[13,14] Therefore, PGE_2 elevation using 15-PGDH inhibitor would be valuable for the management disease that required elevated PGE_2, like wound healing. Wound healing is a complicated process in human or animal in which skin or another organ-tissue repair itself after having wound.[15] In normal skin, epidermis and dermis maintain steady-state equilibrium to maintain protective barrier against the external environment. Once the protective barrier is broken, the wound-healing process immediately set in motion and complex biochemical events takes place to repair the damage.[16] The aim of this study was to screen most potent 15-PGDH inhibitor from plant source and evaluate its wound-healing efficacy using *in-vitro* scratch model in HaCaT cell.

MATERIALS AND METHODS

Materials, reagents, and instruments

Plant extracts were purchased from the Korean Plant Extract Bank (Daejeon, Korea). PGE_2, NAD+, NADH, glutathione-sepharose 4B, dithiothreitol (DTT), sodium dodecylsulfate (SDS), EDTA, reduced dlutathione, mitomycin, and other chemicals and reagents were purchased

Cite this article as: Karna S. *In-vitro* wound healing effect of 15-hydroxyprostaglandin dehydrogenase inhibitor from plant. Phcog Mag 2017;13:122-6.

from Sigma (St. Louis, MO, USA). TGF-β1 was purchased from BioVision (California, USA) and pGEX-2T expression vector from Pharmacia Crop. (New Jersey, USA). cDNA of human 15-PGDH was cloned from human placenta as illustrate earlier.[17] PGE_2 enzyme immunoassay kits were obtained from Thermo Scientific (Rockford, IL, USA). UV-Vis spectrophotometer (Shimadzu, Japan) was used to obtain UV spectra. Real-time PCR was performed using a Light Cycler 2.0 (Roche, Mannheim, Germany). Scratches were visualized and pictures captured using a transmission electron microscope (Hitachi, Tokyo, Japan).

Sample preparation

Different parts of plants were dried in the dark for 7 days and then grounded to powder. The powder samples were extracted three times with ethanol and extracts were obtained through removal of solvents during evaporations. The concentrated samples were stored at -20°C. One hundred milligram of crude extracts were dissolved in 1 ml of commercial grade ethanol.

Expression and purification of 15-PGDH

pGEX-2T expression vector was used to transform 15-PGDH cDNA plasmid into *E. coli* BL-21 lysS. The cells were grown in LB Broth (1.0 L) containing 50 mg/L ampicillin at 37°C and 230 × g. Isopropyl-1-thio-β-d-galactopyranoside was added to 1 mM when OD_{600} reached 0.8 and cells were allowed to grow for additional 12 h at 25°C. Cells were harvested by centrifugation at 4000*g* for 20 min at 4°C and pellets were resuspended in 20 mL of cold cell lysis buffer (1× PBS buffer pH 7.4 containing 1 mM EDTA and 0.1 mM DTT) and sonicated (3 × 15 s at 4°C) to disrupt cells. Sonicated pallets were centrifuged at 4000*g* for 20 min at 4°C and supernatant obtained was applied to a glutathione-sepharose 4B column. The column was rinsed with lysis buffer until OD_{280} reach less than 0.002. 15-PGDH was eluted from column using elution buffer (50 mM Tris-HCl pH 8.0 containing 10 mM reduced glutathione, 1 mM EDTA, and 0.1 mM DTT). The purity of 15-PGDH was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and concentration measured.

15-PGDH assay

The inhibitory activity was evaluated using fluorescence spectrophotometer, measuring formation of NADH at 468 nm following excitation at 340 nm. Reaction mixture was prepared in 2 mL of Tris-HCl buffer (50 mM, pH 7.5) containing 0.1 mM DTT, 0.25 mM NAD^+, purified enzyme (10 μg), 21 μM PGE_2 and various concentration inhibitors. 15-PGDH inhibitory activities were evaluated using standard curve of NADH. Each concentration of inhibitor was assayed in triplicate.

Cell culture, cell viability assay, and determination of PGE_2 release

HaCaT cell line (Keratinocyte cells) was cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat inactivated fetal bovine serum (Sigma) and 100 μg/mL penicillin. Cells were cultured in a 5% CO_2 atmosphere at 37°C. Cell viabilities were evaluated using MTT assay.[18] HaCaT cells (1×10^4/90 μL of DMEM medium) were seeded in 96-well plates. After overnight incubation, plant extract samples were treated for 72 h and after that 10 μL of MTT (5 mg/mL stock solution) was added into each well, incubated for 4 h at 5% CO_2 atmosphere at 37°C. Medium was then removed and 150 μL of DMSO was added to dissolve Formazan crystals. Absorbance was measured at 540 nm using ELISA microplate reader (Perkin-Elmer, California, USA). Extracellular PGE_2 releases were determined in HaCaT cell line using various concentrations of five most potent plant extracts as a 15-PGDH inhibitor. HaCaT cells were seeded (5×10^5 cells/well) into six-well culture plates in DMEM, supplemented with fetal bovine serum and antibiotics for overnight under 5% CO_2, 37°C. Various concentrations of plant extract were added into individual well and media collected after 6 h for extracellular determination and cells were harvested for intracellular determination of PGE_2. Concentrations of PGE_2 were determined using PGE_2 enzyme immunoassay kit (Cayman, MI, USA).

Quantitative real-time PCR

Total cellular RNA was isolated from HaCaT cells according to TRI reagent-specific instruction (RNA iso Plus, Takara Bio. Inc., Shiga, Japan). cDNA was synthesized using reagent-specific instruction of Superscript First Strand synthesis system for reverse transcription-PCR (Invitrogen, Carlsbad, CA, USA) from isolated RNAs. Primers used for real-time PCR were as follows: COX-1 forward, 5'-CCTCAT GTTTGCCTTCTTTGC-3' and reverse, 5'-GGC GGGTACATTTCTCCATC-3' COX-2 forward, 5'-GATCTA CCCTCCTCAA-3' and reverse, 5'-GAACAACTGC TCATCAC-3' MRP4 forward, 5'-AACCTCTAACCGACATTCCTG-3' and reverse, 5'-TCAACATA TTACAGCCACCATC-3' human PGT forward, 5'-GGATGCTGTTTGGAGGAATCCTCA-3' and reverse, 5'-GCACGATCCTGTC TTTGCTGAA-3' and β-actin forward, 5'-GACTATGACTTAGTTGCGTTA-3' and reverse, 5'-GTTGAACTCTCTACATAC TTCCG-3'. PCR reaction mixture contained 4 μL of diluted cDNA (1:5), 10 pmole of each forward and reverse primer, 4 mM $MgCl_2$, and 4 μL of Fast Starter Mix buffer (dNTPs, SYBR Green dye and Tag polymerase).

In-vitro wound healing

In-vitro scratch assay was performed in HaCaT cell line to examine wound-healing effect as previously reported[19,20] using EEAH. HaCaT cells were seeded into six-well plates (5×10^5 cells per well) and grown until reached to 80% confluence. After that media was replaced with serum-free DMEM containing mitomycin (10μg/mL) and cells were incubated for 2 h to prevent wound proliferation. Plates were extensively washed with PBS and then scratches were made using sterile 200 μL pipette tip, and cells were re-washed. TGF-β1 (100 pg/mL) as a positive control or EEAH were added to the medium. Pictures were taken in the same position before and after incubation to document the wound-healing process. Scratch experiments were repeated thrice and representative pictures are included in this study. Scratches were photographed under microscope (×100) immediately after scratching and also after 48 h of incubation at 37°C in 5% CO_2 incubator.

Statistical analysis

Each experiment was performed at least three times and data are expressed as mean ± SE. Statistical significance was determined using paired Student's *t*-test and *P* value less than 0.05 were considered statistically significant.

RESULTS

15-PGDH inhibitory activity assay, cytotoxic assay, and In-vitro PGE_2 determination

15-PGDH inhibitory activities (IC_{50} μg/mL) are illustrated in Supplementary [Table S1]. *Glochidione hirsulta* was the most potent inhibitor for 15-PGDH having 0.36 μg/mL IC_{50}. Five most potent inhibitors were assayed for cytotoxicity. Relative cytotoxicity was determined *in vitro* by anchorage-dependent cells, HaCaT. Result showed that EEAH was more toxic (cytotoxic IC_{50} 70 μg/mL) among tested inhibitor. Further, it is necessary to check whether 15-PGDH inhibitors could increase PGE_2 in cellular or not. Most potent inhibitors were treated for 12 h with concentration of 10 and 100 times of IC_{50} of

Table S1: Plant extracts with potential 15-PGDH-inhibitory activity

Serial No. *	Plant sample	IC_{50} (µg/mL)	Serial No. *	Plant Sample	IC_{50} (µg/mL)
1	*Glochidion hirsulta*	0.37	2	*Glochidion velutinum*	0.51
3	*Cinnamomum tetragonum*	0.52	4	*Memecylon edule*	0.57
5	*Artocarpus heterophyllus*	0.62	6	*Allospondias lakonensis*	0.64
7	*Commersonia batramia*	0.66	8	*Syzygium bullockii aff.*	0.72
9	*Syzygium formosum*	0.73	10	*Choerospondias axillaris*	0.84
11	*Lumnitzera racemosa*	1.31	12	*Osbeckia stellata*	1.52
13	*Trema orientalis*	1.79	14	*Lithocarpus gymnocarpus*	1.92
15	*Planchonella obovata*	2.68	16	*Triumfetta grandidens*	2.87
17	*Triumfetta bartramia*	2.94	18	*Mallotus apelta*	3.19
19	*Baccaurea ramiflora*	3.44	20	*Sonneratia alba*	4.08
21	*Daphniphyllum calycinum*	4.10	22	*Wandlandia paniculata*	4.24
23	*Mucuna pruriens*	4.59	24	*Polygonum hydropiper*	4.94
25	*Macrosolen ochinchinensis*	4.94	26	*Lindra myrrha*	5.47
27	*Melastoma sanguineum*	5.62	28	*Rhodomyrtus tomentosa*	7.56
29	*Melastoma normale*	7.56	30	*Catharanthus roseus*	8.51
31	*Hibiscus tiliaceus*	10.07	32	*Mallotus floribundus*	10.95
33	*Maesa tomentella*	11.83	34	*Machilus velutina*	12.42
35	*Archidendron poilanei*	13.85	36	*Breynia indosinensis*	17.53
37	*Caesalpinia crista*	18.20	38	*Hopea odorata*	19.36
39	*Ipomoea congesta*	42.86	40	*Cleisatanthus tonkinensis*	50.70
41	*Cratoxylum maingayi*	330.30	42	*Gouania leptostachya*	512.54
43	*Engelhardia roxburghina*	573.70	44	*Machilus thunbergii*	916.10
45	*Euodia lepta aff.*	1,170	46	*Senna alata*	2,000
47	*Vitex tripinnata*	2,040	48	*Philydrum lanugiosum*	2,440
49	*Mallotus philippinensis*	2,980	50	*Pandanus humiulis*	3,350
51	*Aidia cochinchinensis*	3,380	52	*Actephila excelsa*	4,040
53	*Aleurites montata*	4,080	54	*Streblus asper*	4,460
55	*Ilex triflora*	5,310	56	*Vernonia cinerea*	6,290
57	*Grewia paniculata*	6,940	58	*Eupatorium odoratum*	7,490
59	*Broussonetia papyrifera*	8,660	60	*Carica papaya*	8,770
61	*Rubus cochinchinensis*	9,160	62	*Helicteres hirsuta*	9,470
63	*Ludwigia epilobioides*	10,050	64	*Desmos chinensis*	10,120
65	*Gomphandra tonkinensis*	10,990	66	*Ficus heteropleura*	11,370
67	*Cerbera manghas*	12,500	68	*Litsea glutinosa*	14,350
69	*Helixanthera parasitica*	14,740	70	*Villebrunea tonkinensis*	15,220
71	*Memecylon umbellatum*	15,520	72	*Ageratum conyzoides*	15,900
73	*Parthenium hysterophorus*	15,950	74	*Spinifix littoreus*	16,480
75	*Celosia argentea*	20,330	76	*Acalypha siamensis*	20,980
77	*Vitex rotundifolia*	22,270	78	*Bridelia monoica*	22,810
79	*Ligustrum sinense*	23,680	80	*Carallia lanceafolia*	24,430
81	*Symplocos cochinchinensis*	25,220	82	*Leucaena leucocephala*	29,960
83	*Ficus hirta*	32,130	84	*Litsea cubeba*	57,250
85	*Ehretia acuminate*	104,200	86	*Aidia oxyodonta*	154,300
87	*Eurya cerasifolia*	992,500	88	*Alocasia odora*	NA
89	*Amaranthus tricolor*	NA	90	*Angelonia goyazensis*	NA
91	*Aporosa tetrapleura*	NA	92	*Archidendron clypearia*	NA
93	*Ardisia quinquegona*	NA	94	*Embelia laeta*	NA
95	*Gymnema sylvestre*	NA	96	*Eurya cerasifolia*	NA
97	*Kibatalia macrophylla*	NA	98	*Hyptis brevipes*	NA

* Potency order of 15-PGDH-inhibitory activity NA: No activity against 15-PGDH

Table 1: Cytotoxic activities of strong 15-PGDH inhibitors and increment of PGE_2 in HaCaT cells

S. No.*	Inhibitor	Cytotoxicity IC_{50} (µg/mL)	% Increment of PGE_2 of the control	
			$10 \times IC_{50}$	$100 \times IC_{50}$
1	*Glochidion hirsulta*	710	50.90	181.84
2	*Glochidion velutinum*	270	27.75	274.92
3	*Cinnamomum tetragonum*	535	39.9	375.65
4	*Memecylon edule*	450	3.55	265.28
5	*Artocarpus heterophyllus*	70	42.03	923.29

*Potency order of 15-PGDH-inhibitory activity

Table 2: Intracellular and extracellular PGE_2 levels after 12-h treatment of tested samples (10 × IC_{50} of 15-PGDH) in HaCaT cells

Sample name	Intracellular (pg/μg) (Mean ± SD)	Extracellular (pg/mL) (Mean ± SD)
Control	1.96 ± 0.18	393.67±12.46
Artocarpus heterophyllus	3.85 ± 0.43*	450.96 ± 15.53*

*Statistically significant $P < 0.05$

Table 3: Extracellular PGE_2 levels (pg/mL) influenced by EEAH in HaCaT cells during *in vitro* wound healing

Time	Sample	Control
0 h	Control	143.6
	EEAH	143.6
12 h	Control	420.36
	EEAH	854.62
24 h	Control	442.86
	EEAH	885.62
48 h	Control	323.28
	EEAH	773.05

15-PGDH inhibition and percentage increment in PGE_2 were measured using ELISA kit. They increased PGE_2 in a concentration-dependent manner and EEAH increased PGE_2 level significantly [Table 1]. Results confirmed that tested inhibitors increased PGE_2 level *in vitro* cellular system. Further investigation was concentrated on EEAH.

Regulation of COX-1/2, PGT, and MRP4 by EEAH

PGE_2 level in cellular system might depend on the expression level of COX-1/2, MRP4, and PGT. COX-1/2 is responsible for the production of PGE_2 from PGH_2 and MRP4 and PGT plays important role in the transportation of PGE_2. Relatively low concentration of extracellular PGE_2 compared with intracellular PGE_2 was observed after the treatment with EEAH, suggesting that above-mentioned factors would determine PGE_2 regulation in biologic systems [Table 2]. HaCaT cells were treated with EEAH and regulation of COX-1/2, MRP4, and PGT was evaluated using real-time PCR. Real-time PCR assay showed that EEAH increased expression level of COX-1/2, MRP4, and PGT [Figure 1]. Observed data showed that high intracellular PGE_2 concentration in HaCaT cells may be due to the activation of COX-1 and 15-PGDH inhibition by EEAH.

Wound-healing effect of EEAH

In-vitro wound healing study was performed using HaCaT scratch model. Various experiment sets were designed to evaluate the results: no drug treatment as a negative control, TGF-β1 (100 pg/mL) as a positive control, EEAH (6.2 μg/mL) only and in combination with COX-1/2 inhibitor (SC 560 0.5μM and Celecoxib 0.5μM), respectively. The photographs were taken before treatment and after 2 days incubation at 37°C, 5% CO_2. EEAH facilitated wound healing (recovery distance: 60%) as compared with the negative control (recovery distance: 15%). Wound-healing effect of EEAH was comparable with TGF-β1 (recovery distance 81%). Similarly, COX-1 inhibitor (SC560) prevented the would-healing effect of EEAH partially (recovery distance: 37%); however, COX-2 inhibitor (Celecoxib, recovery distance 40%) did not interfere with wound-healing effect of EEAH. These results suggested that COX pathway as supplier of PGE_2 plays important role in wound healing [Figure 2]. During wound healing, we have also monitored elevation level of PGE_2 on time interval and higher PGE_2 concentration was observed during 12–24 h and slightly reduced at 48 hr [Table 3].

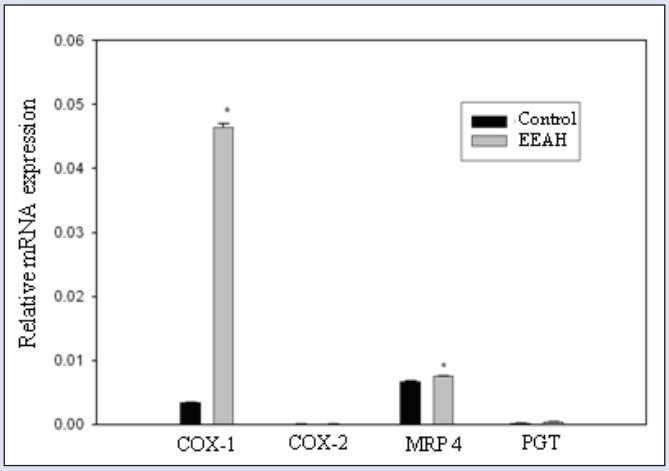

Figure 1: Effects of EEAH on mRNA expression of COX-1, COX -2, MRP4, and PGT * Statistically significant $P < 0.05$

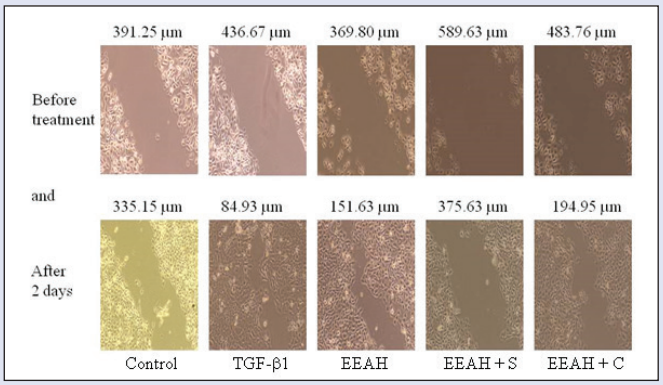

Figure 2: *In vitro* wound-healing effect of EEAH in HaCaT cells. After scratch being made, immediately photographs were taken and widths were measured. Scratched cells were incubated for 2 days in 5% CO_2 incubator, the picture was again captured at the same place and widths were measured. Average width of wound was calculated with longest, medium and shortest length between the both cell populations. Control, no drug treatment; TGF-β1 (100 pg/mL), positive control; EEAH, ethanol extracts of Artocarpus heterophyllus (6.2 μg/mL); SC 560 (0.5 μM); C, Celecoxib (0.5 μM)

DISCUSSION

Plant as a source of medicine used for traditional healthcare system for several thousands of years.[21] New drug development scientists have been focusing their studies on medicinal plants in different parts of the world.[22,23] There are significant economic benefits in the development of new medicines from plants for the treatment of various diseases.[24] Twenty-five percent of medicines belong to plants source and their derivatives.[25] Plants as a source of traditional medicine are used to cure diseases involving skin problems, cold, fever, cough, headache, diarrhea, fertility problems, and toothache.[26] Therefore, 15-PGDH inhibitor was investigated in this study because of diverse therapeutic values and ample of opportunity to develop new drug from plant source. 15-PGDH negatively regulates PGE_2 levels and activity *in vivo*.[27,28] In this study, extracellular and intracellular PGE_2 levels were elevated in HaCaT cell line after treatment of 15-PGDH inhibitors Table 1 and

Table 2. The levels of PGE_2 available to cells are dependent on function of COX-1/2, MRP4, and PGT in PGE_2 signaling. EEAH elevate COX-1 mRNA expression significantly, while MRP mRNA expression slightly, which helps to increase availability of PGE_2 levels in HaCaT cell line [Figure 1]. Recently, Kochel and Fulton reported that MRP4, PGT, and 15-PGDH play important role in regulating PGE_2 levels.[29]

Previously, several scientists have reported wound-healing efficacy of plant extracts.[30–33] This study investigated the role of PGE_2 during the wound-healing process that was induced by plant extract. Tissue regeneration plays important role during recovery from injury, including wound healing, and PGE_2 is candidate molecule that helps in regeneration of tissue.[34] After scratching, extracellular medium was collected on different time interval (0, 12, 24, and 48 h) and PGE_2 concentration was measured. When wound being, the activity of PGE_2 was increased because there was increase on extracellular PGE_2 concentration during 12 and 24 h in all the tested samples because of inflammatory and proliferative actions on wound bed. COX-2 expression and PGE_2 production in the wound bed increase as dermal wound healing transitions from scarless to a scarring phenotype.[12] At a cellular level, PGE_2 was shown to regulate the proliferation of fibroblasts from both wound-healing phenotypes. Hemostasis, inflammatory, proliferative, and remodeling are the major steps of wound healing, where PGE_2 plays an important role.[35] EEAH plays important role to elevate PGE_2 level in cellular system by inhibiting 15-PGDH that accelerates wound healing.

CONCLUSIONS

15-PGDH inhibitor alone will give better result of wound healing than COX inhibitors because of elevated level of PGE_2. Therefore, inhibition of 15-PGDH with plant extracts will be valuable for the therapeutic management of diseases requiring elevated PGE_2 levels like in wound healing. The clinical efficacy and safety of these plant extracts as well as the purification of active ingredients from these plants remains to be done.

Acknowledgement

Plant samples and 15-PGDH plasmid were kindly gifted by Cheol-Hee Choi, Chosun University, Gwangju, Republic of Korea.

Financial support and sponsorship

Nil.

Conflicts of interest

There is no conflict of interests

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