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

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**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 PGE₂ in cellular system. It will be valuable for the therapeutic management of diseases requiring elevated PGE₂ 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 PGE₂ release from HaCaT (Keratinocyte cell line) cell line. Finally, top 15-PGDH inhibitor was selected to evaluate in *vivo* 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 PGE₂ 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 inhibitors. **Results:** 15-PGDH inhibitors elevated PGE₂ levels in concentration-dependent manner. Ethanolic extract of Artocarpus heterophyllus (EEAH), the most potent 15-PGDH inhibitor (IC₅₀ = 0.62 µg/mL) with least cytotoxicity (IC₅₀ = 670 µg/mL), elevated both intracellular and extracellular PGE₂ levels. EEAH facilitated in *vivo* wound healing in a HaCaT (Keratinocyte cell line) scratch model. **Conclusion:** EEAH might apply to treat dermal wounds by elevating PGE₂ levels via COX-1 and 15-PGDH inhibition.

**Key words:** Prostaglandins, cyclooxygenase, PGE₂, wound healing, 15-hydroxyprostaglandin dehydrogenase

**SUMMARY**

- Biological inactivation of 15-PGDH causes elevated level of PGE₂ which will be useful for the management of disease that requires elevated level of PGE₂.

**Tissue injury**

- Arachidonic acid release
- **COX-1**
- **COX-2**
- PGE₂ (i)
- 15-PGDH
- 15-Ketoprostaglandins
- **Plant extract**

**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

**INTRODUCTION**

Prostaglandins (PGs) are lipid compounds that participate in a variety of physiologic and pathologic processes, and among them, PGE₂ is a major mediator for inflammation.¹¹ PGE₂ is formed by PG synthetase from PGH₂ 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 New synthesized PGE₂ is simply diffused and actively extruded by the multidrug resistance 4 (MRP4) from the cells.³ Subsequently, EPR receptor is activated followed by pericellular PGE₂, is cleared via re-uptake of PGE₂ 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 PGE₂ to 15-ketoprostaglandins.⁶

PGE₂ has been known as an important mediator for bone formation,⁷,⁸ gastric ulcer healing,⁹,¹⁰ and dermal wound healing.¹¹,¹² Additionally, PGE₂ has been used to treat gastric ulcer in spite of high price and low efficacy.¹³,¹⁴ Therefore, PGE₂ elevation using 15-PGDH inhibitor would be valuable for the management disease that required elevated PGE₂ 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). PGE₂, NAD⁺, NADH, glutathione-sepharose 4B, dithiothreitol (DTT), sodium dodecylsulfate (SDS), EDTA, reduced glutathione, mitomycin, and other chemicals and reagents were purchased from Sigma-Aldrich Co, USA.

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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.[27] PGE, 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 OD600 reached 0.8 and cells were allowed to grow for additional 12 h at 25°C. Cells were harvested by centrifugation at 4000g 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 pellets were centrifuged at 4000g 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 OD280 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 PGE2 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 PGE2 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% CO2 atmosphere at 37°C. Cell viabilities were evaluated using MTT assay.[18] HaCaT cells (1 × 104/90 µL of DMEM medium) were seeded (5 × 105 cells/well) into six-well culture plates in DMEM, supplemented with fetal bovine serum and antibiotics for overnight under 5% CO2, 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 PGE2. Concentrations of PGE2 were determined using PGE2 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'-CCTCATGGTGGCCTTCTTTGC-3' and reverse, 5'-GGCGGTTATTTTCTCCATC-3' COX-2 forward, 5'-GATCTCTCCCTCAAA-3' and reverse, 5'-GAACACCTGCTCATCAG-3' MRP4 forward, 5'-AACGCTCAACGCATTGCTG-3' and reverse, 5'-TCAAATATACTACCTACCCA-3' human PGT forward, 5'-GGATGCTGTCTGGAGGAATTCTCCA-3' and reverse, 5'-GCCAGATCCTGTCTTGTGCATTGAA-3' and β-actin forward, 5'-GACTAGTACTTATGGTGCGTTA-3' and reverse, 5'-GTGGAAACTCTCTGATACAC-3'. PCR reaction mixture contained 4 µL of diluted cDNA (1:5), 10 pmole of each forward and reverse primer, 4 mM MgCl2, 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[18,26] using EEAH. HaCaT cells were seeded into six-well plates (5 × 104 cells/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% CO2 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 PGE2 determination
15-PGDH inhibitory activities (IC50 µg/mL) are illustrated in Supplementary [Table S1]. Glochidione hirsuta was the most potent inhibitor for 15-PGDH having 0.36 µg/mL IC50. 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 IC50 70 µg/mL) among tested inhibitor. Further, it is necessary to check whether 15-PGDH inhibitors could increase PGE2 in cellular or not. Most potent inhibitors were treated for 12 h with concentration of 10 and 100 times of IC50 of
**Table S1**: Plant extracts with potential 15-PGDH-inhibitory activity

| Serial No. * | Plant Sample                  | IC<sub>50</sub> (µg/mL) | Serial No. * | Plant Sample                  | IC<sub>50</sub> (µg/mL) |
|--------------|-------------------------------|--------------------------|--------------|-------------------------------|--------------------------|
| 1            | Glochidion hirsulta           | 0.37                     | 2            | Glochidion velutinum          | 0.51                     |
| 3            | Cinnamomum tetragonum         | 0.52                     | 4            | Memecylon edule               | 0.37                     |
| 5            | Artocarpus heterophyllus      | 0.62                     | 6            | Allopondias lakanensis        | 0.64                     |
| 7            | Commersonia bartrami         | 0.66                     | 8            | Syzygium bullockii            | 0.72                     |
| 9            | Syzygium formosum            | 0.73                     | 10           | Choerospondias assilis        | 0.84                     |
| 11           | Lumnatzeria 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 grandiflora       | 2.87                     |
| 17           | Triumfetta bartrami          | 2.94                     | 18           | Mallotus apelta              | 3.19                     |
| 19           | Baccaraea 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 tomentosae       | 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 pallanei       | 13.85                    | 36           | Breynia indosinensis         | 17.53                    |
| 37           | Caesalpinia crista           | 18.20                    | 38           | Hopea odorata                | 19.36                    |
| 39           | Ipomea congesta             | 42.86                    | 40           | Cleisanthus tonkinensis       | 50.70                    |
| 41           | Cratoxylum maingayi          | 330.30                   | 42           | Gouania leptostachya         | 512.54                   |
| 43           | Engelhardia roxburghiana    | 573.70                   | 44           | Machilus thunbergii          | 916.10                   |
| 45           | Euodia lepta aff.           | 1.170                    | 46           | Senna alata                  | 2,000                    |
| 47           | Vitex triplinata            | 2.040                    | 48           | Philedrum lanagiosum         | 2,440                    |
| 49           | Mallotus philippinensis     | 2.980                    | 50           | Pandanus humilis             | 3,350                    |
| 51           | Aidia cochinchinensis       | 3.380                    | 52           | Actephila excelsa            | 4,040                    |
| 53           | Aleurites montata           | 4.080                    | 54           | Streblus asper               | 4,460                    |
| 55           | ilx 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 cochinensis           | 9.160                    | 62           | Heliocrates 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 mahaghas            | 12.500                   | 68           | Litsea glutinosa             | 14,350                   |
| 69           | Helxanthera parasitica      | 14.740                   | 70           | Villebrunea tonkinensis      | 15,220                   |
| 71           | Memecylon umbellatum        | 15.520                   | 72           | Ageratum conyzoides          | 15,900                   |
| 73           | Parthenium hystrophorus     | 15.950                   | 74           | Spinifex littoreus           | 16,480                   |
| 75           | Celosia argentea            | 20.330                   | 76           | Acalypha siamensis           | 20,980                   |
| 77           | Vetex rotundifolia          | 22.270                   | 78           | Bridelia monoica             | 22,810                   |
| 79           | Ligustrum sinense           | 23.680                   | 80           | Carallia lanceaefolia        | 24,430                   |
| 81           | Symplacos cochinchinensis   | 25.220                   | 82           | Leucaena leucocephala        | 29,960                   |
| 83           | Ficus hirta                 | 32.130                   | 84           | Litsea cubeba                | 57,250                   |
| 85           | Ebreia acuminate            | 104.200                  | 86           | Aidia oxyoodontia           | 154,300                  |
| 87           | Eurya cerasifolia           | 992.500                  | 88           | Alclosia odorata             | 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           | Gymnemem sylvestre          | NA                       | 96           | Eurya cerasifolia            | NA                      |
| 97           | Kibatulial 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<sub>2</sub> in HaCaT cells

| S. No.* | Inhibitor                  | Cytotoxicity IC<sub>50</sub> (µg/mL) | % Increment of PGE<sub>2</sub> of the control |
|---------|----------------------------|--------------------------------------|----------------------------------------------|
|         | 10 × IC<sub>50</sub>       | 100 × IC<sub>50</sub>                |                                              |
| 1       | Glochidion hirsulta        | 710                                  | 50.90                                        |
| 2       | Glochidion velutinum       | 270                                  | 27.75                                        |
| 3       | Cinnamomum tetragonum      | 535                                  | 39.9                                         |
| 4       | Memecylon edule            | 450                                  | 3.55                                         |
| 5       | Artocarpus heterophyllus   | 70                                   | 42.03                                        |

* Potency order of 15-PGDH-inhibitory activity
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]. These results 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%); however, COX-2 inhibitor (Celecoxib, recovery distance 40%) did not interfere with wound-healing effect of EEAH.

**DISCUSSION**

Plant as a source of medicine used for traditional healthcare system for several thousands of years. New drug development scientists have been focusing their studies on medicinal plants in different parts of the world. There are significant economic benefits in the development of new medicines from plants for the treatment of various diseases. Twenty-five percent of medicines belong to plants source and their derivatives. Plants as a source of traditional medicine are used to cure diseases involving skin problems, cold, fever, cough, headache, diarrhea, fertility problems, and toothache. 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. In this study, extracellular and intracellular PGE$_2$ levels were elevated in HaCaT cell line after treatment of 15-PGDH inhibitors Table 1 and

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**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 | EEAH |
|------|--------|---------|------|
| 0 h  | Control| 143.6   | 143.6|
| 12 h | Control| 420.36  | 854.62|
| 24 h | Control| 442.86  | 885.62|
| 48 h | Control| 323.28  | 773.05|

**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)
Table 2. The levels of PGE₂ available to cells are dependent on function of COX-1/2, MRP4, and PGT in PGE₂ signaling. EEAH elevate COX-1 mRNA expression significantly, while MRP mRNA expression slightly, which helps to increase availability of PGE₂ levels in HaCaT cell line [Figure 1]. Recently, Kochel and Fulton reported that MRP4, PGT, and 15-PGDH play important role in regulating PGE₂ levels.[30-32] Previsously, several scientists have reported wound-healing efficacy of plant extracts.[18-20] This study investigated the role of PGE₂ during the wound-healing process that was induced by plant extract. Tissue regeneration plays important role during recovery from injury, including wound healing, and PGE₂ is candidate molecule that helps in regeneration of tissue.[33] After scratching, extracellular medium was collected on different time interval (0, 12, 24, and 48 h) and PGE₂ concentration was measured. When wound being, the activity of PGE₂ was increased because there was increase on extracellular PGE₂ concentration during 12 and 24 h in all the tested samples because of inflammatory and proliferative actions on wound bed. COX-2 expression and PGE₂ production in the wound bed increase as dermal wound healing transitions from scarless to a scarring phenotype.[12] At a cellular level, PGE₂ 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 PGE₂ plays an important role.[34] EEAH plays important role to elevate PGE₂ 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 PGE₂. Therefore, inhibition of 15-PGDH with plant extracts will be valuable for the therapeutic management of diseases requiring elevated PGE₂ 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.

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Conflicts of interest

There is no conflict of interests

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