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RESEARCH ARTICLE

Mechanistic Insights into *Uncaria gambir* Ethanol Extract's Modulation of Cytoglobin and ECM Protein in Keloid Fibroblasts

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SUMMARY

Keloids are benign tumors that result from abnormal wound healing. These growths may cause cosmetic and functional problems, especially when they grow in the joint area. Although the pathogenesis is not fully understood, stimulated fibroblasts cause excess collagen deposition in the extracellular matrix, leading to keloid formation. They also synthesize cytoglobin, the overexpression of which can protect against the fibrotic process. At present, keloid therapy remains unoptimized, primarily because keloids often experience recurrence after treatment. Therefore, studies to find keloid therapy are still being carried out. This study explored the effect of ethanol extract of Uncaria gambir (EG) on the expression of cytoglobin, elastin, desmosine, and collagen type I, and III of keloid fibroblasts. Fibroblasts were isolated from keloid and non-keloid skin tissues using primary culture techniques. An enzyme-linked immunosorbent assay (ELISA) method was used to determine the expression of cytoglobin, elastin, and desmosine. Meanwhile, immunocytochemistry was used to determine collagen I and III expression. The results showed that EG increased the expressions of cytoglobin, elastin, and desmosine while reducing the expression of collagen types I and III. EG is likely to have potential as an anti-keloid.

Key Words: *Cytoglobin, extracellular matrix, fibroblast, keloid, Uncaria gambir.*

Uncaria gambir Etanol Ekstresinin Keloid Fibroblastlarda Sitoglobin ve ECM Proteininin Modülasyonuna İlişkin Mekanik İçgörüler

ÖZ

Keloidler, anormal yara iyileşmesi sonucu oluşan iyi huylu tümörlerdir. Özellikle eklem bölgesinde büyüdüklerinde kozmetik ve fonksiyonel sorunlara neden olabilmektedir. Patogenezi tam olarak anlaşılmamış olsa da uyarılmış fibroblastlar, ekstraselüler matrikste aşırı kollajen birikimine neden olarak keloid oluşumuna yol açmaktadır. Ayrıca, aşırı eksprese edilmesi fibrotik sürece karşı koruma sağlayabilen sitoglobini de sentezlerler. Günümüzde keloid tedavisi hala optimize edilememiştir, çünkü keloidler genellikle tedaviden sonra sıklıkla tekrarlar. Bu nedenle, keloid tedavisini bulmaya yönelik araştırmalar devam etmektedir. Bu çalışmada, Uncaria gambir (EG) etanol ekstresinin keloid fibroblastlardaki sitoglobin, elastin, desmosin ve tip I ve III kollajen ekspresyonu üzerindeki etkisi araştırılmıştır. Fibroblastlar, primer kültür teknikleri kullanılarak keloid ve keloid olmayan deri dokularından izole edilmiştir. Sitoglobin, elastin ve desmosin ekspresyonunu belirlemek için enzim bağlantılı immünosorbent testi (ELISA) yöntemi kullanılmıştır. Öte yandan, kollajen I ve III ekspresyonunu belirlemek için immünositokimya kullanılmıştır. Sonuçlar, EG'nin sitoglobin, elastin ve desmosin ekspresyonunu artırırken, kollajen tip I ve III ekspresyonunu azalttığını göstermiştir. EG'nin anti-keloid potansiyele sahip olduğu düşünülmektedir.

Anahtar Kelimeler: *Sitoglobin, hücre dışı matris, fibroblast, keloid, Uncaria gambir.*

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INTRODUCTION

During wound healing, neutrophils and macrophages release transforming growth factor (TGF)-1 and platelet-derived growth factor (PDGF), activating fibroblasts to differentiate into myofibroblasts, which produce extracellular matrix (ECM) components, primarily collagen (Andrews et al., 2016). Myofibroblasts express α-SMA and nonmuscle myosins, enhancing contractility (Shinde et al., 2017). Under normal conditions, they undergo apoptosis, regulated by interleukin-1 (IL-1), fibroblast growth factor-1 (FGF-1), and prostaglandin E2 (PGE2). However, failed apoptosis leads to prolonged myofibroblast activity, disrupting ECM composition and contributing to keloid formation (Cohen et al., 2017; Hinz & Lagares, 2020). Collagen provides structural strength, while elastin, rich in desmosine (DES) and other amino acids, maintains skin elasticity (Baumann et al., 2021; Leiva et al., 2018; Schräder et al., 2018).

Excess ECM deposition characterizes keloids, leading to persistent fibrotic growth beyond the original wound margins (Andrews et al., 2016; Nangole & Agak, 2019). Studying keloid pathology *in vivo* is challenging, as keloids do not develop in experimental animals, making *in vitro* fibroblast cultures a widely accepted alternative for investigating keloid-associated cellular and molecular mechanisms. Previous studies have demonstrated that fibroblasts isolated through primary culture retain their phenotype according to the tissue sources, supporting the relevance of this model (Deng et al., 2021; Ningsih et al., 2024; Qin et al., 2021).To provide a comparative baseline, non-keloid fibroblasts (NKFs) from surrounding skin are used as controls, as previous studies have shown that they offer a reliable reference for assessing fibroblast behavior and ECM composition in keloid research (Ningsih et al., 2024; Qin et al., 2021).

Fibroblasts expressed cytoglobin (CYGB), a vertebrate globin protein expressed in various tissues (Burmester et al., 2002; Nakatani et al., 2004). While its function remains unclear, CYGB is regulated by the hypoxia-inducible-factor-1α (HIF-1α) protein, which enhances its expression under hypoxia. Inhibiting HIF-1α with ibuprofen reduced CYGB expression in keloid fibroblast (KF) (Jusman et al., 2019), and siRNA-mediated CYGB inhibition impaired mitochondrial biogenesis and function in KFs (Jusman et al., 2021). CYGB is also involved in oxygen distribution and free radical scavenging under hypoxic conditions (Guo et al., 2007; Jusman et al., 2014; Mathai et al., 2020; Xu et al., 2006). Furthermore, CYGB overexpression exhibits antifibrotic effects in the liver, kidney, and ocular tissues (Mimura et al., 2010; Wei et al., 2019; Xu et al., 2006).

Keloids frequently recur despite available treatments, highlighting the need for alternative therapies (Sutheno, 2021). Gambir (*Uncaria gambir* [W.Hunter] Roxb.), a plant rich in polyphenolic compounds, is widely cultivated in West Sumatra and possesses anti-lipid peroxidation, antibacterial, antiseptic, and antioxidant properties (Dewi & Pratiwi, 2018; Fasrini & Lipoeto, 2021; Jusman et al., 2022; Melia et al., 2015; Ningsih et al., 2014). Recent studies suggest its therapeutic potential. Ethanolic extract of gambir (EG) has hepatoprotective effects in a carbon tetrachloride (CCl_4) induced liver injury (Fahrudin et al., 2015) and prevents bleomycin-induced pulmonary fibrosis by inhibiting nuclear factor kappa beta (NF-κB), transforming growth factor beta (TGF-β), tissue inhibitor metalloproteinase (TIMP)-1, and collagen type-I (COL I) formation (Desdiani et al., 2022). Additionally, the *in-silico* analysis revealed that gambirin A1, procyanidin B2, and neooxygambirtannine—components found in EG—can bind to PDGF-A, potentially inhibiting its activity (Jusman et al., 2022). These findings provide insight into possible molecular mechanisms of EG in modulating keloid fibroblast proliferation. This study examines the effects of EG on CYGB and ECM components (elastin, desmosine, collagen I/III) in keloid fibroblasts. While CYGB is not an established fibrosis marker, it has been reported to have antifibrotic potential, particularly through oxi-

dative stress regulation. Investigating its expression in response to EG may provide new insights into keloid pathophysiology. ECM markers were selected as they are key indicators of fibrosis progression (Deng et al., 2021; Limandjaja et al., 2020).

MATERIAL AND METHODS

Study design

The study adopted an experimental design using KFs as the primary culture. We obtained keloid tissues from three women at the Tanjung Priok Hospital in Jakarta, Indonesia, whose previous Caesarean section wounds had developed keloids and who had undergone another Caesarean section procedure. The KFs were divided into an untreated control group and groups treated with different concentrations of EG. This study also used the normal keloid fibroblasts (NKFs) cultured in a complete medium without EG as an additional control. The NKFs used in this study were isolated from normal tissue around keloid wounds. All the keloid tissues were acquired with the patient's consent. The study was conducted from June 2021 to February 2022 at the Department of Biochemistry and Molecular Biology and the Department of Histology, Faculty of Medicine, Universitas Indonesia. The Faculty of Medicine Universitas Indonesia Research Ethics Commission approved this study's ethics evaluation with the number 472/UN2. F1/ETIK/2016.

Uncaria gambir extraction

The ethanol extract of gambir used in this study was obtained from Dr. Sri Ningsih of the Health Research Organization, Research Center for Pharmaceutical Ingredients and Traditional Medicine (BRIN). The gambir, sourced from a community product in West Sumatra, Indonesia, was identified at the Biology Department of the Indonesian Institute of Sciences (LIPI) Cibinong, Indonesia, and herbarium specimen has been deposited at LIPI with the reference number 1085/IPH.1.02./If.8/VI/2013.

To prepare the extract, the whole part of the *Uncaria gambir* plant was steamed and then pressed to obtain the sap. The sap was left overnight, dried in an oven at 45–50°C, and further ground. A 25 g dried gambir sap was soaked in ethanol and shaken for 24 hours before filtration. The resulting filtrates were then separated and evaporated under a vacuum at 45°C until a semisolid mass was formed. To obtain the dried extract, all samples were further dried in an oven at 40°C for 24 hours.

Fibroblast isolation

We isolated the KFs and NKFs using explant cultures, as described in our previous study (Ningsih, et al., 2024; Siregar et al., 2019). All the isolation procedures were conducted in a biological safety cabinet (BSC) II. Keloid and nonkeloid tissues were chopped into smaller fragments (1 × 1 × 1 mm) before being cultured in a 24-well plate with a complete culture medium consisting of low glucose DMEM (Cat No. 11885084, Gibco, New York, USA), 10% FBS (Cat No. A4766901, Gibco, New York, USA), 1% amphotericin (Cat No. 15290026, Gibco, New York, USA), and 1% penicillin-streptomycin (Cat No. 15140122, Gibco, New York, USA). The tissues were incubated at 37°C with 5% CO_2. Cells that reached 80% confluence were harvested using Tryple Select (Cat No. 12563029, Gibco, New York, USA) and counted using an automated LUNA cell counter. Flow cytometry with CD73+, CD105+, and CD90+ marker antibodies (BD Stemflow, New Jersey, USA) was used to examine the fibroblast population. The fibroblasts used in this study were from passages 4 to 6.

EG treatment of KF cultures

The EG was dissolved in ethanol at a concentration of 1000 ppm. The working concentrations of EG for KF treatment were determined based on the IC_{50} test from our previous study (Jusman et al., 2022), which were 12.5 and 50 μg/mL. Before application, the extract was diluted in a complete culture medium to achieve the final working concentration, ensuring that the ethanol concentration remained at a level that did not affect cell viability. The KFs were grown in culture at a density of $3x10^5$ cells/well in a complete me-

dium. They were incubated for 24 hours at 37°C with 5% CO_2. After that, the cells were treated with EG at 12.5 and 25 µg/mL for another 24 hours. The total protein from each group of cells was isolated and then analyzed for research parameters such as All analyses of CYGB [Cat No. MBS2533318, Mybiosource, San Diego, USA], ELN [Cat No. MBS2021199 Mybiosource, San Diego, USA], and DES [Cat no. MBS730011, San Diego, USA] were performed using sandwich ELISA according to the manufacturer's protocol.

Isolation of proteins from KFs and NKFs

For total protein analysis, KF cells treated with various concentrations of EG and nonkeloid fibroblast cells were extracted and isolated using a mammalian protein extraction reagent (Cat No. 78505, Thermo-Fisher, Rockford, IL, USA). The total protein concentration was measured using a Bradford assay (Cat No. 5000006, Bio-Rad, CA, USA).

Immunocytochemistry analysis of COL I and COL III

COL I and COL III were measured using an immunocytochemistry method based on that used in our previous study (Nauli et al., 2023). First, at a density of 1.5 x 10^3 cells/well, KFs and NKFs were cultured in a complete medium for 72 hours at 37^0 C with 5 % CO_2. After 72 hours, the KF cells were treated with EG at different concentrations for 24 hours. The cells were incubated with a 1:100 diluted primary antibody of COL I (Invitrogen, Catalog No. PA1-2604, Rockford, USA) and COL III (Cat No. PA1-28870, Invitrogen, Rockford, USA) for 2 hours. After that, the cells were incubated for 1 hour in a 1:400 dilution of IgG-HRP antibody (Cat No. A1949, Sigma-Aldrich, Saint Louis, USA). Along with hematoxylin, 3,3'-diaminobenzidine (DAB) was used to stain the cells. Microphotographs of each group were documented using Optilab Viewer 3.0.

Statistical analysis

The data obtained were analyzed using GraphPad Prism version 10.1.0. We presented the data from triplicate experiments in mean and standard deviation (SD) values. We used a one-way analysis of variance (ANOVA) followed by least significant differences (LSD) as a post hoc test for normally distributed data. Statistical significance was set to $\alpha = 5\%$, a 95% confidence interval (CI), and a *P*-value of < 0.05.

RESULTS

Cytoglobin concentration

CYGB concentrations were significantly lower in KFs (176 ± 10.1 pg/mg protein) than in NKFs (639 ± 9.44 pg/mg protein) (Figure 1). The addition of EG 12.5 µg/mL (212 ± 10.5 pg/mg protein) and 25 µg/mL (235 ± 8.73 pg/mg protein) significantly increased CYGB concentration in keloid fibroblasts compared to the absence of EG (176 ± 10.1 pg/mg protein). Similarly, CYGB concentrations were significantly higher in fibroblasts treated with EG 25 µg/mL than EG 12.5 µg/mL. The result indicated that CYGB concentration in KFs rose as the EG concentration increased (ANOVA, LSD test, $p < 0.05$).

Elastin concentration

The addition of EG 12.5 µg/mL (140 ± 1.021 pg/mg protein) and 25 µg/mL (157 ± 1.074 pg/mg protein) to KFs increased ELN protein concentration significantly compared to EG-untreated KFs (120 ± 0.972 pg/mg protein). ELN concentrations in fibroblasts treated with EG 25 µg/mL were significantly higher than those treated with EG 12.5 µg/mL. Like CYGB, the concentration of ELN in KFs increased with the rising concentration of EG used (Figure 2). Nevertheless, ELN concentrations in all KF treatment groups remained significantly lower compared to the NKF group.

Desmosine concentration

Significant differences in DES concentration were found between all groups, demonstrating that KF contained substantially less DES than NKF (Figure 3). When EG 12.5 µg/mL (173 ± 3.85 pg/mg protein) and 25 µg/mL (207 ± 6.14 pg/mg protein) were added to keloid fibroblast cultures, DES levels increased significantly compared to KF without EG addition (157 ± 2.21 pg/mg protein) (ANOVA, LSD test, $p < 0.05$).

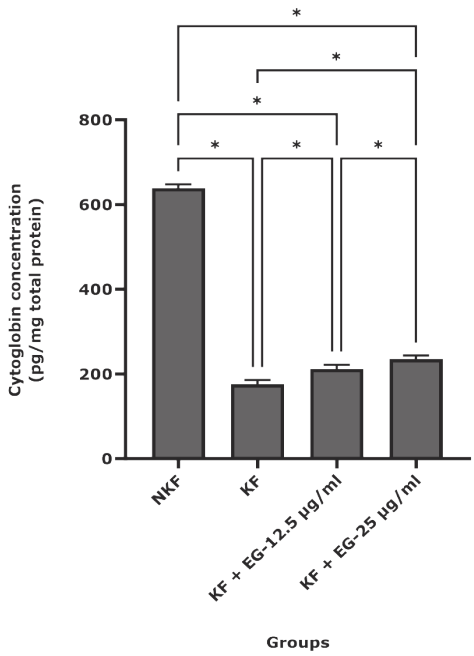

Figure 1. Cytoglobin concentrations in nonkeloid (NKF) and keloid fibroblasts (KF) without and with the addition of various concentrations of EG (ANOVA, LSD test, *P<0.05; n = 3).

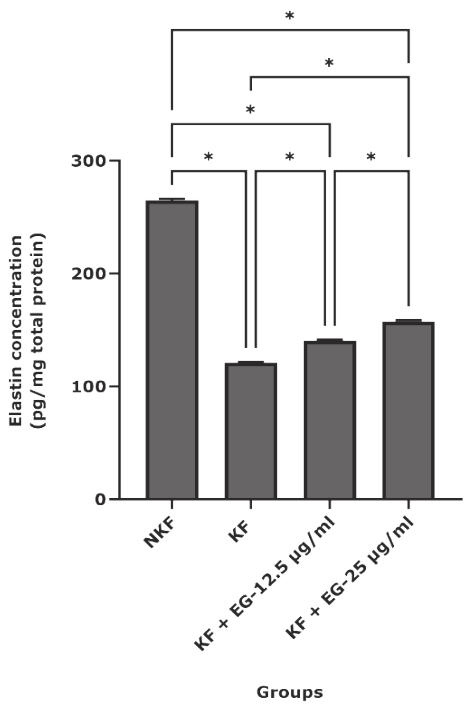

Figure 2. Concentrations of elastin in nonkeloid (NKF) and keloid fibroblasts (KF) with and without the addition of EG at various concentrations (ANOVA, LSD test, *P<0.05; n = 3).

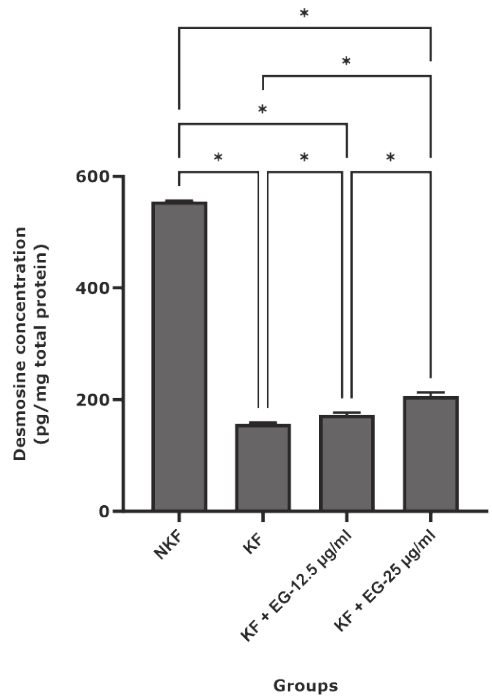

Figure 3. Desmosine concentrations in nonkeloid (NKF) and keloid fibroblasts (KF) in the absence and presence of varying concentrations of EG. (ANOVA, LSD test, *P<0.05; n = 3).

COL I and COL III distribution

In the NKF, each fibroblast expressed COL I, indicated by the brownish granular appearance of the cell membrane and adjacent extracellular areas (Figure 4A). These results were also found in the KFs (Figures 4B–D). The COL I expression patterns in the EG-treated groups (at both 12.5 µg/mL and 25 µg/mL concentrations) were different from those observed in the untreated group, and the EG-treated groups showed predominantly intracytoplasmic COL I expression (Figures 4C and 4D). COL I expression in KF treated with 12.5 µg/mL EG distinctly showed more prominent COL I (Figure 4C) than in KF treated with 25 µg/mL EG (Figure 4D).

In the NKF cultures, fibroblasts expressed COL III, indicated by the brownish granular appearance of the cytoplasm, membrane, and adjacent extracellular areas (Figure 4E). These results were also found in KF, which expressed COL III predominantly in the extracellular area (Figures 4F–H). COL III expression

in the EG-treated groups showed different expression levels at 12.5 µg/mL and 25 µg/mL concentrations. The 12.5 µg/mL EG-treated group showed intracytoplasmic COL III expression (Figure 4G), while the 25 µg/mL EG-treated group showed minimal to no COL III expression (Figure 4H). Based on the result, KF treated with 12.5 µg/mL EG clearly showed more prominent COL III (Figure 4G) compared to KFs treated with 25 µg/mL EG (Figure 4H).

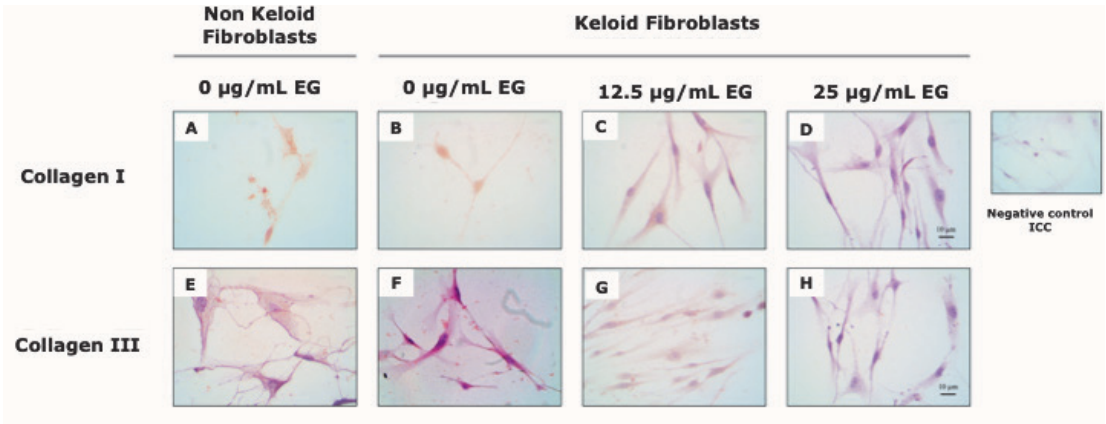

Figure 4. Immunocytochemistry of COL I and III from nonkeloid fibroblast culture (A &E); keloid fibroblast culture without treatment (B&F); keloid fibroblast culture treated with 12.5 µg/mL EG (C&G); and keloid fibroblast culture treated with 25 µg/mL EG (D&H). These microphotographs were taken as representative microphotographs from each group random five high power field microphotographs using Optilab. The scale bar represents 10 µm. Negative control is shown in the right upper side.

Discussion

This study examined the effect of EG on KFs and found that EG at 12.5 and 25 µg/mL increased CYGB levels in KFs compared to untreated KFs, although levels remained lower than in NKFs. CYGB, known for its antifibrotic properties, reduces ROS, leading to decreased α-smooth muscle expression and COL I levels (Dat et al., 2021; Hieu et al., 2022; Lv et al., 2018; Okina et al., 2020). CYGB's antioxidant role helps lower oxidative stress, as shown by reduced kidney nitrotyrosine accumulation with CYGB overexpression (Mimura et al., 2010).

In keloids, increased activation and proliferation of fibroblasts cause elevated ROS, leading to excessive collagen production, inflammation, and fibrosis (Desdiani et al., 2020; Siregar et al., 2019). EG may help reduce ROS and fibrosis in keloids by increasing CYGB expression, which has ROS-scavenging properties. Our previous study showed that CYGB expression has a negative correlation with ROS levels in KFs (Siregar et al., 2019). EG's effect on CYGB expression may involve the TGF-β signaling pathway. Okina et al. found that TGF- β reduced CYGB expression in hepatic stellate cells through the pSMAD2/SP-3 M1 pathway, leading to oxidative stress and liver fibrosis (Okina et al., 2020). In silico studies by Desdiani et al. showed that components of *U. gambir* (e.g., (+)-catechin, epigallocatechin gallate, procyanidin B3) had strong binding affinities with TGF- β, potentially blocking its receptor (Desdiani et al., 2020). Thus, EG may prevent TGF- β from binding to its receptor, increasing CYGB expression (Desdiani et al., 2020). Therefore, the presence of EG components may prevent TGF-β from binding to its receptor and cause increased CYGB expression.

The results showed that EG treatment (12.5 and 25 µg/mL) increased ELN expression in KFs compared to untreated KFs. The mechanism behind this increase is unclear but may involve the inhibition of fibroblast activation into myofibroblasts, leading to higher matrix metalloproteinase (MMP) activity, particularly MMP-9. This stabilizes ELN in the extracellular matrix, as MMP-9 is known to degrade ELN. The study also found that DES amino acid expression increased

with ELN, as DES is a key component of ELN (Ozsvar et al., 2021). DES forms cross-links with other amino acids like AA, IDES, and LNL to create ELN. According to Ozsvar et al., tropoelastin, an ELN precursor, cross-links with lysine and DES through the action of lysyl oxidase (LOX), which is crucial for ECM renewal (Ozsvar et al., 2021).

Immunocytochemistry revealed differences in COL I and III distribution in KFs, suggesting that EG may inhibit the secretion of COL I from the cytoplasm to the extracellular matrix. Higher EG concentrations (12.5 and 25 μg/mL) showed greater inhibition of COL I and III expression. This may be due to EG's ability to inhibit the TGF-β signaling pathway, which regulates ECM components like collagen (Tong et al., 2019). EG components may bind to TGF-β receptors, thus influencing collagen expression (Desdiani et al., 2020).

Additionally, EG may reduce collagen deposition by inhibiting the PDGF-α/PDGFR-α signaling pathway. A previous *in silico* study showed that EG components like gambirin A1, procyanidin B2, and neooxygambirtannine could bind to PDGFA, potentially inhibiting PDGF activity (Jusman et al., 2022). Since fibrosis involves excessive ECM formation and deposition (Klinkhammer et al., 2018), and PDGF/PDGFR signaling plays a key role in fibrosis development (Olson & Soriano, 2009), inhibiting this pathway may help prevent fibrosis.

Overall, EG (12.5 and 25 μg/mL) significantly increased CYGB, ELN, and DES expression while reducing COL I and III secretion in KFs. These ECM changes, along with CYGB upregulation, may contribute to fibrosis modulation, suggesting EG's potential as a treatment for keloid fibrosis. However, this study has several limitations. The collagen secretion was assessed using immunocytochemistry, which provides localization data but lacks precise quantification. Future studies should incorporate biochemical validation of EG's active compounds and quantitative protein analysis to further clarify its antifibrotic mechanisms. The proposed mechanism of *U. gambir* ethanol extract in inhibiting keloid in this study is illustrated in Figure 5.

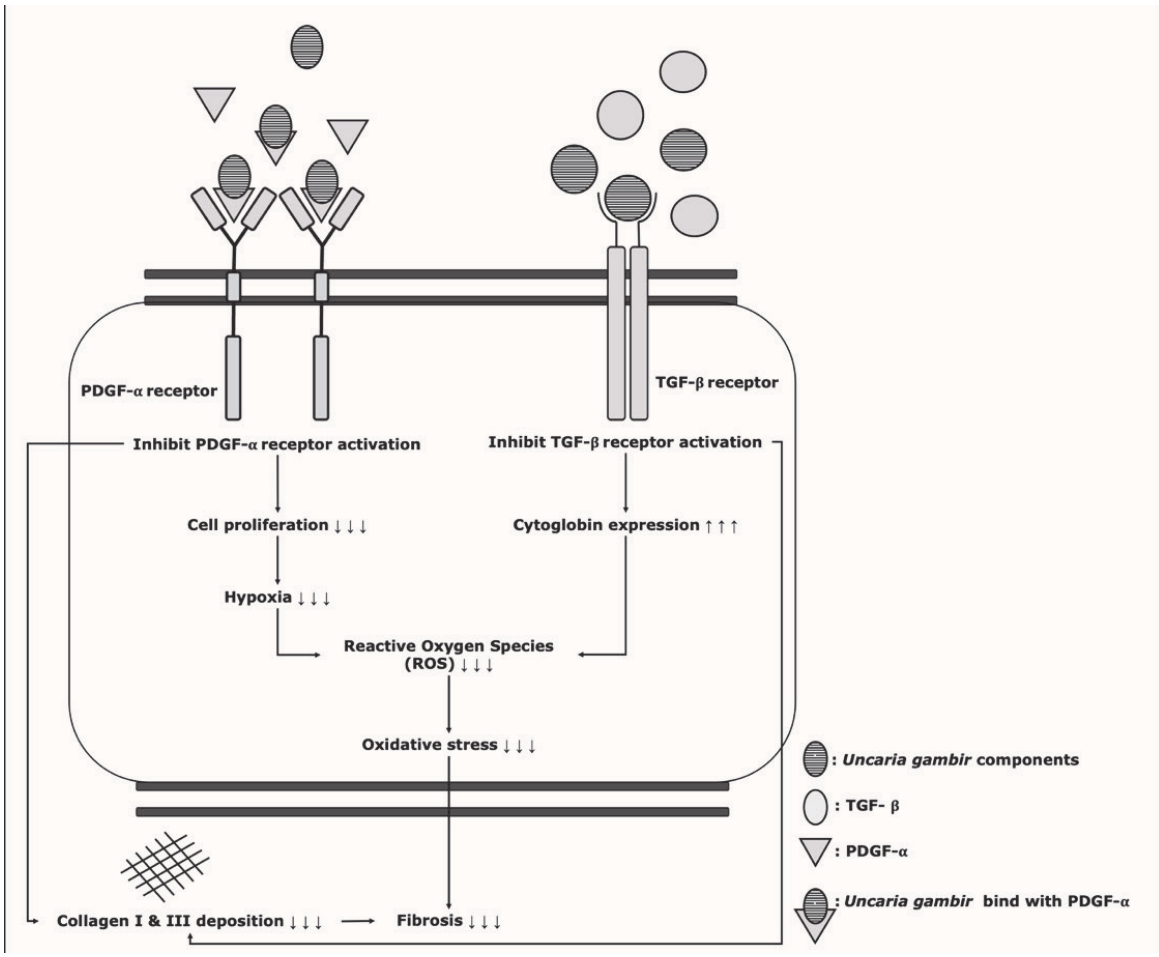

Figure 5. The proposed mechanism of *Uncaria gambir* inhibits fibrosis in keloid through PDGF and TGF-β

CONCLUSION

Treatment with 12.5 µg/mL and 25 EG significantly increased CYGB, ELN, and DES expression in KFs but decreased COL I and COL III secretion from the cytoplasm to extracellular areas in KFs. Therefore, EG is a potential candidate for the future treatment of keloids.

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AUTHOR CONTRIBUTION RATE STATEMENTS

SWAJ: Study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, contributing to the completion of the manuscript, obtained funding, study supervision; MFA: Acquisition of data, analysis, and interpretation of data, drafting the manuscript, statistical analysis, contributing to the completion of the manuscript; RN: Acquisition of data, analysis, and

interpretation of data, critical revision of the manuscript, technical and administrative support, contributing to the completion of the manuscript; RP: Technical support, contributing to the completion of the manuscript, study supervision; SN: Material support, contributing to the completion of the manuscript; RDA: Technical and material support, study supervision; contributing to the completion of the manuscript.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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