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Unraveling Potential Compounds of *Uncaria gambir* (W.Hunter) Roxb. as Antikeloid Agent: In Silico, In Vitro and Ex Vivo Experimental Validation

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Abstract

Background/Objectives: Keloid treatment remains challenging due to limited effectiveness and patient dissatisfaction. Herbal-based therapy offers promising alternatives that require further investigation. *Uncaria gambir* (W.Hunter) Roxb., an original plant from Indonesia, possesses an antifibrotic effect. However, its potential as an antifibrotic agent in keloid management remains unclear. This study aims to bridge this gap by evaluating the bioactive compound from gambir and its effects on keloid fibroblast primary culture. Methods: The bioactive compounds of gambir extract and fractions (ethanol, hexane, and ethyl acetate fractions) were identified by using liquid chromatography–mass spectrometry (LCMS/MS) analysis. The mechanism of gambir bioactive compounds for keloid was predicted using the compound–protein interaction network and enrichment analysis, and validated using molecular docking and dynamic simulation. The experimental study results, including cytotoxic and bioactivity effects, were represented as IC_{50} and selectivity index (SI) values, and the ex vivo analysis of keloid tissue explants. Results: Uncariagambiriine was identified as the most potent compound with the lowest binding energy and high stability to the core protein targets: AKT1 and TGFB1. The ethanol fraction was determined to have the highest abundance of gambir's typical bioactive compounds, with the lowest IC_{50} (128.76 ± 0.24 µg/mL) and the highest SI (6.32) value. Furthermore, the results of the ex vivo analysis indicated the significant inhibition of keloid fibroblast proliferation and migration by the gambir ethanolic fraction. Conclusions: This study underlines the potential of the gambir ethanolic fraction as an antifibrotic agent in keloid, warranting further investigation and development for clinical applications.

Keywords: gambir; bioactive compounds; bioactivity; keloid

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1. Introduction

Keloid is characterized by an abnormal fibroproliferative wound-healing process beyond the boundaries of the original wound. This fibroproliferative disorder may emerge after any kind of dermal injury, leading to cosmetic and physiological issues that affect a patient's quality of life. The current treatment approaches for keloids are still facing formidable challenges due to high recurrence rates and dissatisfaction [1]. Natural compounds derived from medicinal plants offer a wide range of benefits, including minimal side effects and a diversity of bioactive compounds. The diversity of bioactive compounds may be found in the plant extract or fractions attributed to the complex interactions, which perform synergistic therapeutic effects. The use of these synergistic effects can maximize the therapeutic efficacy, with minimal adverse effects, through multiple compounds and multiple target properties [2,3]. Therefore, exploring novel therapeutic agents derived from natural sources is required to unravel their potential therapeutic benefits against keloid.

Uncaria gambir (W.Hunter) Roxb., a plant species belonging to the Rubiaceae family, has garnered attention for its diverse pharmacological properties, including anti-inflammatory, antioxidant, anticancer, and antifibrotic activities [4–6]. This plant is originally from Indonesia and is widely found in West Sumatera. Gambir has long been employed as a traditional medicine for various ailments [7]. Munggari et al. [8] revealed that gambir contains secondary metabolites belonging to the flavonoid, phenolic, and alkaloid groups. Catechin and its derivatives have been recognized as the major flavonoids in gambir, exhibiting various biological effects, including antioxidant activity through the inhibition of lipid peroxidation [9] and analgesic effects in a rat model of osteoarthritis [10]. Unfortunately, the information regarding the antifibrotic properties of Gambir's metabolites remains limited and fragmented. Previously, in vivo studies by Desdiani et al. [11] and Sriningsih [12] demonstrated the potential of gambir in attenuating pulmonary and hepatic fibrosis via an in vivo study. Therefore, gambir is proposed to have the same effect on skin fibrosis, particularly keloid. Supporting this hypothesis, Jusman et al. [13] reported that gambir extract inhibited the growth of KF with Gambiriin A1, procyanidin B2, and neooxogambirtannine, identifying it as a potent inhibitor of PDGF via an in silico study. Those groups of bioactive compounds are dissolved well in polar solvents like ethanol or methanol. Furthermore, the separation of bioactive compounds by fractionation is required to obtain a group of target compounds that have a high efficiency against fibrosis.

The pathomechanism of keloid relies on cell-to-cell interaction mediated by growth factors. The growth factors contribute to excessive keloid fibroblast proliferation and the overproduction of extracellular matrix components. Notably, keloid fibroblasts exhibit increased expression of growth factors and their receptors, leading to heightened cell proliferation, enhanced cell survival, and excessive synthesis of extracellular matrix (ECM) proteins, particularly collagen [14]. Our previous study identified gambiriin C, isogambirine, and procyanidin B1 as promising compounds targeting transforming growth factor beta 1 (TGFβ1), AKT serine/threonine kinase 1 (AKT1), and matrix metallopeptidase 1 (MMP1). Further gene ontology enrichment and pathway analyses predicted that gambir bioactive compounds may regulate cell proliferation, migration, transcription, and signal transduction via profibrotic cytokine and growth factor signaling pathways [15].

Experimental validation of the potential effects of gambir bioactive compounds on keloid remains unclear. To address this gap, this study investigates the effects of gambir ethanolic extract and its fractions on keloid-derived fibroblasts obtained from explant primary culture. The primary cell culture systems offer a valuable model for closely mimicking the in vivo microenvironment [16]. Keloid fibroblasts are recognized as the key drivers of keloid pathogenesis due to their elevated proliferative activity and increased synthesis of ECM proteins, particularly collagen [17]. To comprehensively evaluate the

effects of gambir's bioactive compounds on keloid fibroblasts, this research conducted fractionation of the gambir ethanolic extract using solvents of varying polarity. The fractions exhibit simpler compound profiles, which facilitate the identification of the most potent active compounds with inhibitory activity against keloid fibroblasts [18]. We focus on the bioactivity, cytotoxicity, and inhibitory effects of keloid-derived fibroblast primary culture. By elucidating the effects of Gambir-derived compounds on keloid fibroblasts, this study aims to advance understanding of their therapeutic potential in keloid management.

2. Materials and Methods

2.1. Extraction and Fractionation Procedure

Dry plant powder derived from the whole part of the gambir plant was used as the sample for this research. The sample was purchased from a local farmer from Pesisir Selatan, West Sumatera, Indonesia, and identified as *Uncaria gambir* (W.Hunter) Roxb. by herbarium dekopensis, Department of Biology, Universitas Indonesia (JI25-P-66). The sample was extracted by using the maceration technique with 96% ethanol (Merck, 107017, Darmstadt, Germany) as the solvent. 950 mg of the dry plant powder of gambir was weighed and then extracted using 96% ethanol (1:1) at room temperature for 24 h. Subsequently, the crude extract was fractionated using liquid–liquid separation method using solvents with different polarities. Ten grams of gambir ethanol extract were dissolved in 100 mL of ethanol (Merck, 107017, Darmstadt, Germany) and transferred to a separatory funnel. An equal volume of hexane (Merck, 100795, Darmstadt, Germany) was added, and the mixture was shaken for 5 min and left to stand for 30–60 min until phase separation occurred. The upper hexane layer was collected, and 100 mL of ethyl acetate (Merck, 100789, Darmstadt, Germany) was added to the remaining solution. After repeating the mixing and settling steps, the ethyl acetate (lower) and ethanol (upper) fractions were separated. Each fraction was then concentrated, followed by drying and weighing to calculate the percentage of the yields using the following formula:

$$Yield\ (\%) = \frac{Weight\ of\ extract\ or\ fraction\ obtained}{Weight\ of starting\ material} \times 10$$

2.2. Phytochemical Analysis

Phytochemical analysis was performed by subjecting the gambir extract and fraction to total phenol and flavonoid content. The total phenolic content was determined by following the Folin–Ciocalteu method. The absorbance was assessed through the standard curve 0, 50, 100, 150, 200, 250, and 300 ppm of gallic acid for phenolic and quercetin for flavonoid. The absorbance was examined using a GENESYS™ 150 UV-Vis Spectrophotometer (Thermo Scientific, 840-309400, Waltham, MA, USA) at 765 nm for phenolic [19] and 376 nm for flavonoid [20].

2.3. Gambir Bioactive Compound Screening Process

An ultra-performance liquid chromatography (UPLC) system (LC: ACQUITY UPLC® H-Class System, Waters, Milford, MA, USA) coupled to a mass spectrometer (Xevo G2-S QTof, Waters, Milford, MA, USA) was used for LCMS/MS analysis. Chromatographic separation was performed using a C18 column (1.8 µm 2.1 × 100 mm, ACQUITY UPLC® HSS, Waters, Milford, MA, USA) at a flow rate of 0.2 mL/min, and the column oven temperature was set at 50 °C. The mobile phase consisted of water +5 mM ammonium formic acid and acetonitrile +0.05% formic acid. The sample injection volume was 5 µL (initially filtered through a 0.2 µm syringe filter), with a total run time per sample was 23 min. The liquid chromatography (LC) analysis employed a mobile phase with a specific flow rate, while the mass spectrometry (MS) analysis utilized electrospray ionization (ESI)

in positive mode within a defined mass range of 50–1200 m/z and source and desolvation temperatures of 100 and 350 °C, respectively. Correspondingly, cone and desolvation gas flow rates were set at 0 L/h and 793 L/h, along with variable collision energy varied between 4 and 60 eV. Data acquisition, analysis, and instrument control were facilitated using Masslynx software version 4.1 [21].

2.4. In Silico Analysis

The chemical compounds and their canonical Simplified Molecular Input Line Entry System (SMILE) of gambir ethanolic extract and fractions from LCMS/MS data were collected from https://pubchem.ncbi.nlm.nih.gov (accessed between 6 and 8 October 2024). The SMILES numbers were used as keywords to identify potential targets for keloid in the SwissTargetPrediction database http://swisstargetprediction.ch (accessed on 20 October 2024), with human genes set as data retrieval. The keloid target proteins were sourced from our previous study [15]. The Gambir compounds and keloid target proteins interaction network was constructed, visualized, and analyzed using Cytoscape 3.9.1 software on 22 October 2024. The core proteins from the previous steps were imported into the Database for Annotation, Visualization, and Integrated Discovery (DAVID) https://david.ncifcrf.gov/ (accessed on 27 October 2024) for functional and pathway analysis. The analysis was conducted with an adjusted p value less than 0.05 as the cut-off value for indicating statistical significance.

The protein structure of the target protein for molecular docking analysis was retrieved from the Protein Data Bank (PDB) database https://www.rcsb.org/ (accessed on 5 November 2024), while the structures of active compounds as the ligand were sourced from PubChem (accessed on 5 November 2024). Molecular docking of gambir bioactive compounds with target proteins was performed using AutoDockTools 1.5.6 on 9 November 2024. The molecular docking simulation utilized the Lamarckian Genetic Algorithm. The docking score's absolute value was used to determine the binding affinity and stability between the compounds and their targets. Visualization of the molecular docking results was carried out using Discovery Studio 4.5 on 15 November 2024. The stability of compound-target binding was simulated by molecular dynamic analysis using YASARA software v. 25.12.1 and run with the md_run.mcr file. The molecular dynamics simulation was conducted with a pH of 7.4, using Na and Cl ions, at a temperature of 298 K, with a duration of 30 ns (30,000 ps) and a save interval of 10 ns (10,000 ps). The Amber14 force field was applied as specified in the md_run.mcr file. The radius of gyration (Rg), root mean square deviation (RMSD) of protein, and root mean square fluctuation (RMSF) of amino acid residues were used to evaluate the compound–protein interaction [22].

2.5. Cytotoxic and Bioactivity Effect

Cytotoxic and bioactivity effects were performed using keloid and normal fibroblast primary cultures from fresh keloid tissue based on our previous study [23]. The sample derived from surgical excision was donated for research purposes only with written informed consent. The procedure was approved by the ethics committee of the Faculty of Medicine, Universitas Indonesia—Cipto Mangunkusumo Hospital (No: KET-1206/UN.F1/ETIK/PPM.00.02/2023). The cytotoxic evaluation was performed by using the CCK-8 cell counting kit (Dojindo, CK04, Kumamoto, Japan) assay procedure in various doses (μg/mL) and represented as the IC_{50} value (the sample concentration that causes 50% inhibition or cell death). In brief, 5000 fibroblast cells derived from passage 2 were cultured in 96 well-culture plates, then incubated in a CO_2 incubator at 37 °C and 5% CO_2 for 24 h. The fibroblast cell will adhere to the bottom of the dish after 24 h. Afterwards, the media was replaced with 100 μL of treatment media with duplicates for each treatment. The

treatment media were made from gambir crude extract, ethanol, ethyl acetate, and hexane fractions with various concentrations. Dimethyl sulfoxide (DMSO) has been employed as the solvent and optimized at a non-cytotoxic concentration, ensuring the effects were due to the gambir treatments. 100 µL of complete media (DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic) was added for the negative control and the blank well. The plate was then incubated in an incubator at 37 °C and 5% CO_2 for 24 h. The old media was discarded after 24 h, then rinse each well with 100 µL of sterile Ca and Mg-free Phosphate-Buffered Saline (PBS) (Gibco, 18912-014, Grand Island, NY, USA). Next, the complete media was added to each well, and continued with 10 µL of CCK-8 solution was added to each well. The plate was covered with aluminum foil and placed into a CO_2 incubator at 37 °C and 5% CO_2 for four hours. The absorbance was read at wavelength 450 nm using a microplate reader, Multiscan FC (Thermo, 357, Waltham, MA, USA), then used for determining the cell viability. The percentage of cell viability was calculated using the following formula:

$$\%\ cell\ viability = \frac{(OD\ (control - blank) - OD\ (treated - blank))}{OD\ (control - blank)}$$

The absorbance of treated refers to the optical density (OD) measured in wells treated with the test compounds, while the absorbance of control refers to wells containing untreated cells (negative control). The viability value was employed for measuring IC_{50} values. This value was obtained from the graphs of percentage inhibition versus concentration of Gambir extract and fraction. Furthermore, the selectivity index (SI) was determined as a simple ratio between the IC_{50} of normal and keloid fibroblasts, defined as follows. The treatment with the lowest IC_{50} value and the highest SI will be selected for the ex vivo assay.

$$SI = \frac{IC50\ normal\ fibroblast}{IC50\ keloid\ fibroblast}$$

2.6. Ex Vivo Assay Analysis

The dermal fragment of keloid tissue was placed into the bottom of 24 well culture plates. Leave the plate semi-opened for 10–15 min in a type 2 biosafety cabinet (BSC) to facilitate the fragment adhering. 200 µL of complete media was added into each well by placing the plate into a CO_2 incubator at 37 °C and 5% CO_2 for 24 h. After 24 h of incubation, the media were replaced with 200 µL of treatment media. The treatment media was carried out as the cytotoxicity and bioactivity assay with the dose based on the IC_{50} value, $\frac{1}{2}\times$ IC_{50}, $1\times$ IC_{50}, and $2\times$ IC_{50} value. In this study, positive control was not included due to the currently limited understanding of Gambir's mechanism of action in keloids. Therefore, it is not feasible to select a positive control with a comparable mode of action. Accordingly, the effects of treatments were assessed relative to untreated keloid tissue explant as a negative control to establish a baseline response. All treatments and negative control (complete media only) were plated out in quadruplets. A representative figure was captured at the same location on days 3 and 6. At the end of incubation (day 6), the cell was counted by using a fluorescent imaging assay using 4′,6-diamidino-2-phenylindole (DAPI)-aqueous fluoroshield (Abcam, ab104139, Waltham, MA, USA). The fluorescence image was acquired using an inverted fluorescence microscope (ZOE Fluorescent Cell Imager-Bio-Rad, Bio-Rad, Hercules, CA, USA) under standardized settings. Cell quantification was performed by analyzing the images with ImageJ 1.54g apps, which allowed for accurate counting of DAPI-stained nuclei to estimate cell numbers.

3. Results

3.1. Phytochemical and Bioactive Compounds Analysis of Gambir

The brown solid masses were yielded from dry plant powder of gambir extraction using ethanol as the solvent. The crude extract was then fractionated and yielded three fractions with different structures and colors. The pale brown solid mass was yielded from the ethanol fraction, the hexane fraction was solid and deep brown in color, while a reddish-brown semi-solid mass was obtained from the ethyl acetate fraction. Either the crude extract or fractions of Gambir positively contained phenolic and flavonoid properties in this study (Table 1). The ethanol fraction has the highest total phenol and flavonoid content compared to the other fractions, although the total flavonoid content of the crude extract remains the highest value compared to the fractions. In parallel with the result, gambir's typical bioactive compounds were highly abundant in this fraction. Table 2 represents the typical bioactive compounds that have been identified in gambir extract and fractions (Supplementary Materials). This study identified procyanidin B1, gambiriin A1, dihydrogambirtannine, uncariagambiriine, and oxogambirtannine as gambir's typical bioactive compounds found in gambir extract and fractions. Those bioactive compounds have been previously reported as major constituents of gambir extracted using ethanol or ethyl acetate as the solvent [8].

Table 1. Total phenolic and flavonoid contents of gambir's crude extract and fraction.

Gambir's Sample	Yield (%)	Total Phenolic (mg GAE/g)	Total Flavonoid (mg QE/g)
Crude extract	44	33.4	19.59
Ethanol Fraction	10.95	34.1	17.09
Hexane Fraction	25.61	34.0	12.39
Ethyl Acetate Fraction	1.14	33.4	13.33

Table 2. LC-MS analysis of Gambir extract and fraction.

Sample	RT (min)	Abundance (%)	*m/z*	Formula	Suspect Compound
Crude extract	3.41	1.91	579.15	C30H26O12	Procyanidin B1
	4.27	0.35	581.17	C30H28O12	Gambiriin A1
	6.95	2.23	333.16	C21H20N2O2	Dihydrogambirtannine
	7.28	14.46	621.22	C36H32N2O8	Uncariagambiriine
Ethanol fraction	1.28	0.97	579.15	C30H26O12	Procyanidin B1
	4.29	0.36	581.16	C30H28O12	Gambiriin A1
	7.01	4.50	333.16	C21H20N2O2	Dihydrogambirtannine
	7.26	8.27	621.22	C36H32N2O8	Uncariagambiriine
	8.53	1.96	345.12	C21H16N2O3	Oxogambirtannine
Ethyl acetate fraction	1.28	0.85	579.15	C30H26O12	Procyanidin B1
	4.29	0.34	581.16	C30H28O12	Gambiriin A2
	7.01	3.61	333.16	C21H20N2O2	Dihydrogambirtannine
	7.26	7.10	621.22	C36H32N2O8	Uncariagambiriine
	8.51	2.73	345.12	C21H16N2O3	Oxogambirtannine
Hexne fraction	6.99	4.30	333.16	C21H20N2O2	Dihydrogambirtannine
	7.24	8.19	621.22	C36H32N2O8	Uncariagambiriine

3.2. In Silico Analysis

The interaction between keloid targets and identified gambir's bioactive compounds from previous analysis is illustrated in Figure 1a. The edges connecting the nodes highlight the potential multi-target effects of Gambir's compounds on keloid targets. The intensity of the element's color correlates with the number of interactions between compounds and target proteins, the darker shades indicating a higher degree of interaction. Figure 1b complements the interaction network by illustrating the result of gene ontology (GO) enrichment analysis, highlighting the biological processes (BP), molecular functions (MF), and cellular components (CC) associated with the biological targets. The size of the dots

represents the count of genes involved in each category, while the color gradient from green to red corresponds to the significance level (-log10(*p*-value)), with red indicating higher statistical significance.

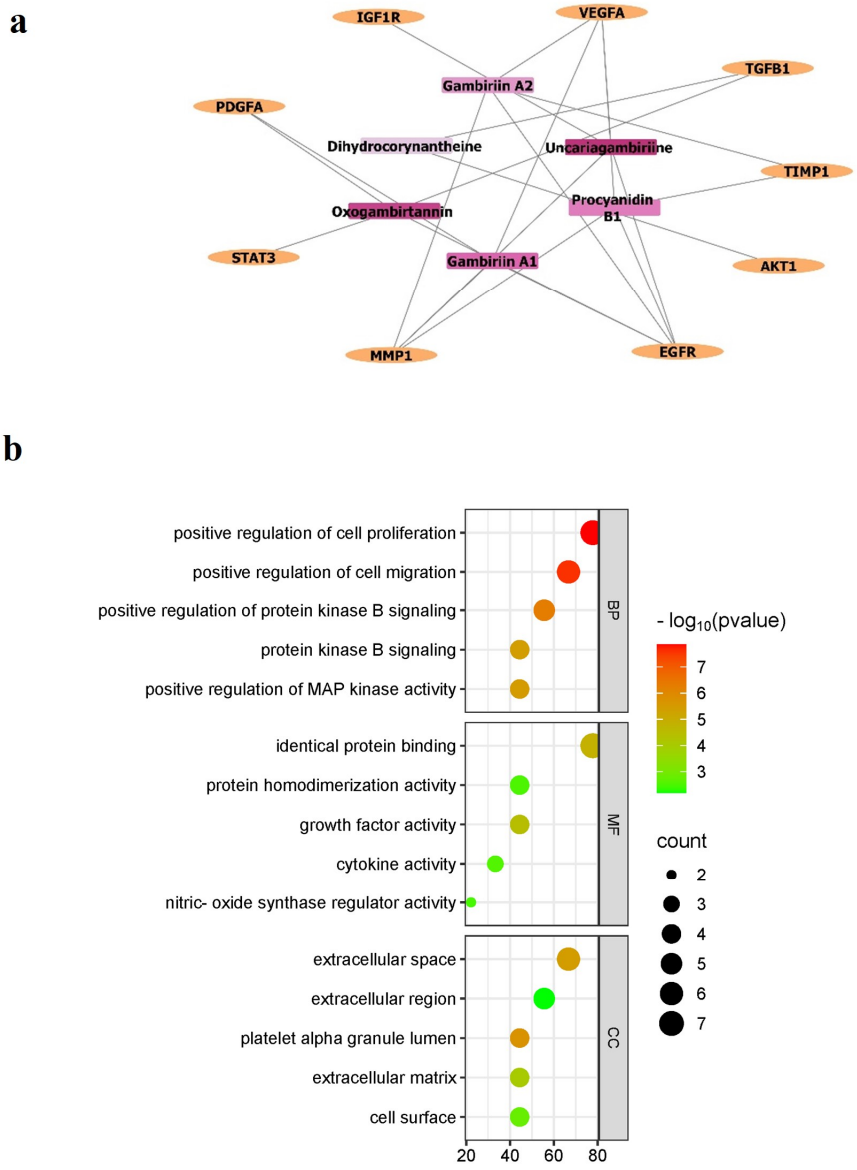


Figure 1. (**a**) Drug–protein interaction network and (**b**) Gene ontology enrichment analysis. Pink: Gambir bioactive compounds and yellow: Keloid target proteins. Biological process (BP), molecular function (MF), and cellular components (CC) related to the core target proteins.

The molecular docking analysis was conducted to validate the interaction between the active compounds of gambir (Table 2) and the keloid target proteins. This study focused on two target proteins: TGFβ1 (3KFD) and Akt1 (4GV1); these proteins belonged to the key targets with the highest interaction scores (based on our previous study [15]) and were involved in the same signaling pathway, which is the non-canonical TGFβ pathway. This molecular docking test is shown in Table 3 and visualized in Figure 2. A lower binding affinity of energy suggests a more stable connection between the protein and its ligand [24]. The binding affinities of the test compound were then compared to the binding affinities of the target proteins and their native ligands. Each target protein possesses a native ligand that is positioned within the active site of the protein, 0XZ501 defined as the native ligand for TGFβ1 (3KFD) and L01EX27 for Akt1 (4GV1).

Table 3. The molecular docking analysis of Gambir's bioactive compounds with keloid target proteins.

Gambir Bioactive Compounds	Binding Affinity (Kcal/mol)	
	TGFβ1	Akt
Procyanidin B1	−8.05	−9.33
Gambiriin A1	−6.03	−9.17
Gambiriin A2	−5.41	−10.51
Dihydrogambirtannine	−6.75	−9.18
Uncariagambiriine	−8.24	−11.02
Oxogambirtannine	−7.16	−8.84
Native ligand	−7.21	−10.73

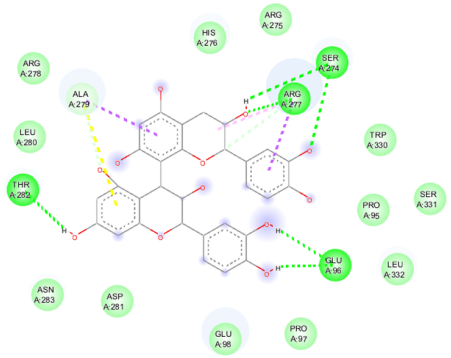
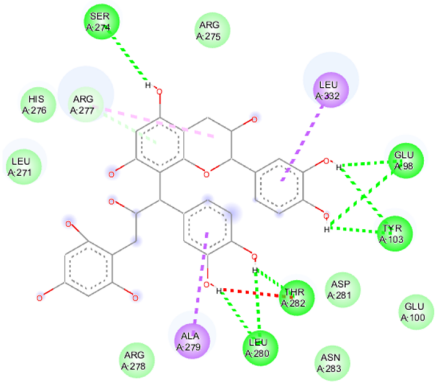
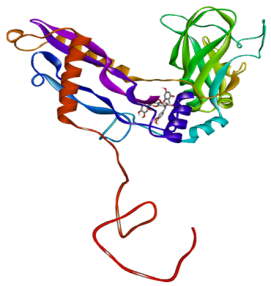
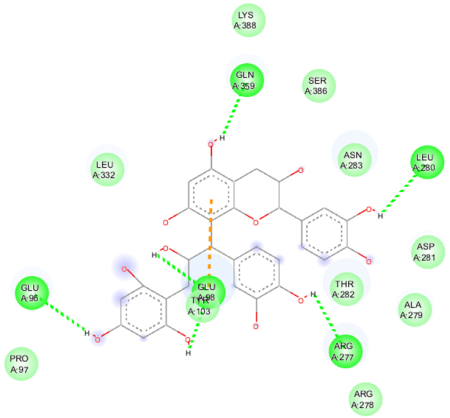
Protein-ligand complex	3D	2D
TGFβ1-procyanidin B1		
TGFβ1-gambiriin A1		

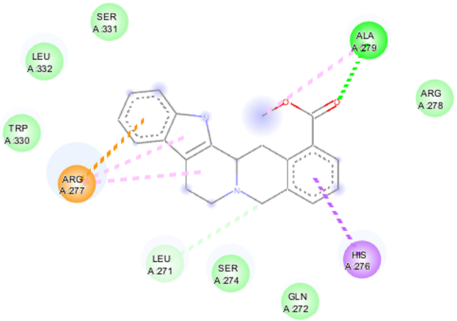
Figure 2. *Cont.*

TGFβ1-gambiriin A2

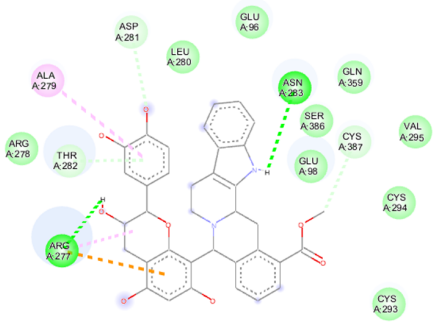




TGFβ1-dihydrogambirtannine



TGFβ1-uncariagambiriine



TGFβ1-oxogambirtannine

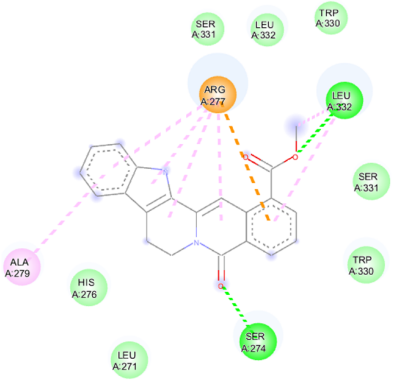
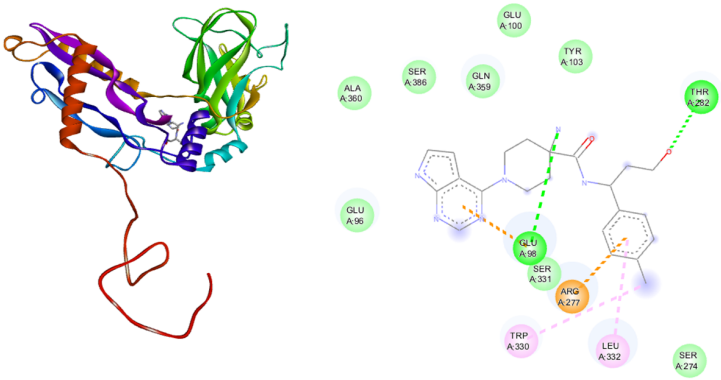
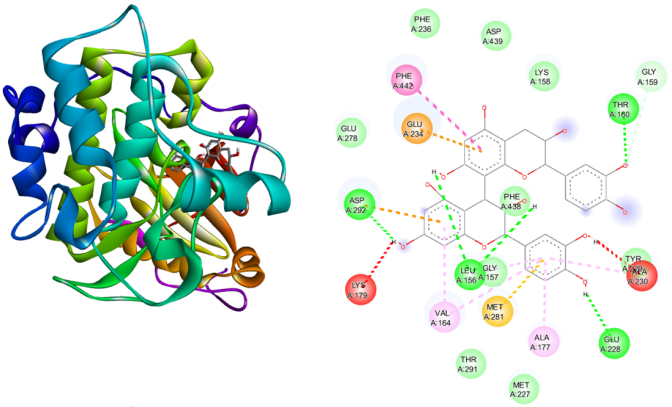


Figure 2. *Cont.*

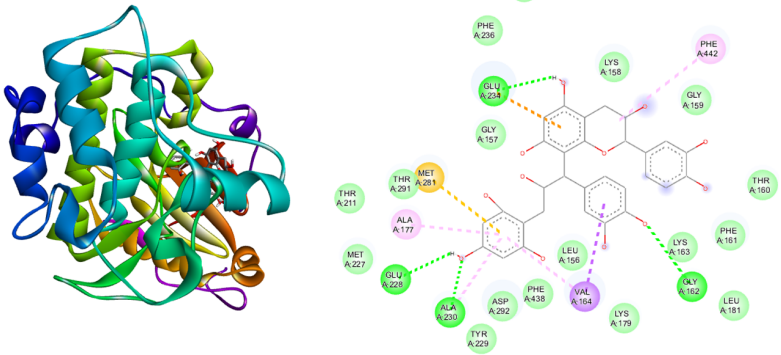
TGFβ1-native ligand



AKT-procyanidin B1



AKT-gambiriin A1



AKT-gambiriin A2

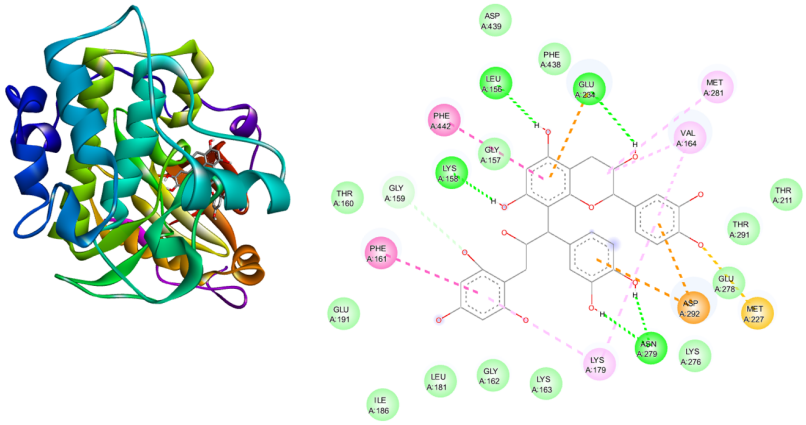


Figure 2. *Cont.*

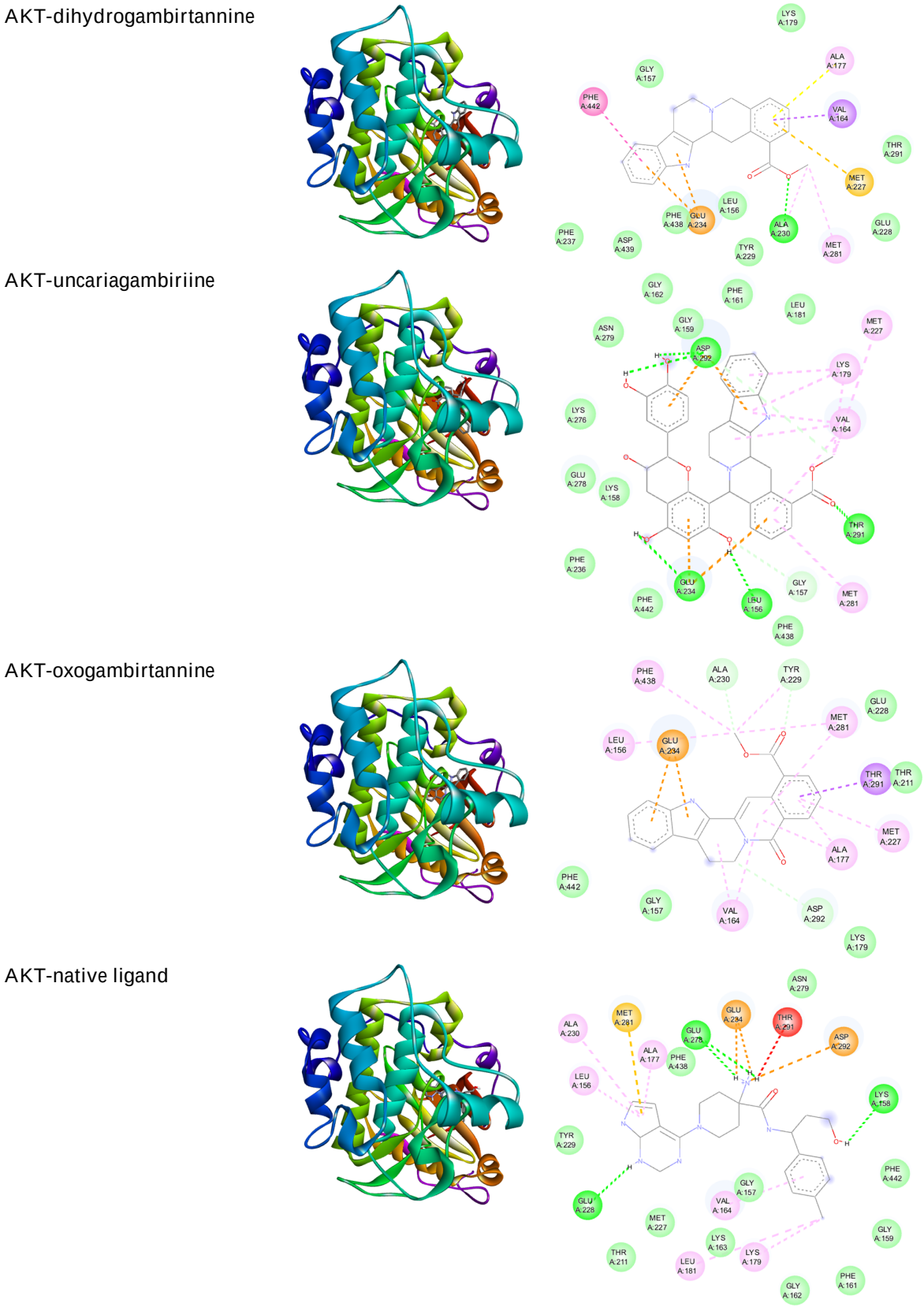


Figure 2. Molecular docking visualization of uncariagambiriine-TGFβ1 and uncariagambiriine-Akt complex. The dashed lines represent the specific interaction type; green: conventional hydrogen bonds, light green: carbon-hydrogen bonds; light purple: pi-alkyl interactions; violet: pi-sigma interaction; orange: pi-anion interaction; red: unfavorable donor–donor interaction.

The molecular dynamics simulation data indicates that the solute achieves structural stability after an initial equilibration phase (Figure 3). The radius of gyration (Rg) stabilizes around 33.5–34.0 Å in TGFβ1 and 21.7–22.0 Å in Akt1 after slight compaction during the first 8 ns. The ligand movement RMSD relative to the receptor steadily increases in the first 3 ns and is relatively stable during 4–15 ns. The value starts to fluctuate after 15 ns until the end of the simulation, suggesting significant positional adjustments or weakened binding interactions over time. The ligand conformation RMSD indicated dynamic conformational flexibility after 8 ns. The solute residue RMSF highlights distinct regions of flexibility and rigidity, reflecting functional or interaction-related dynamics. These results suggest stable solute behavior alongside flexible ligand dynamics, which may impact ligand-receptor interactions.

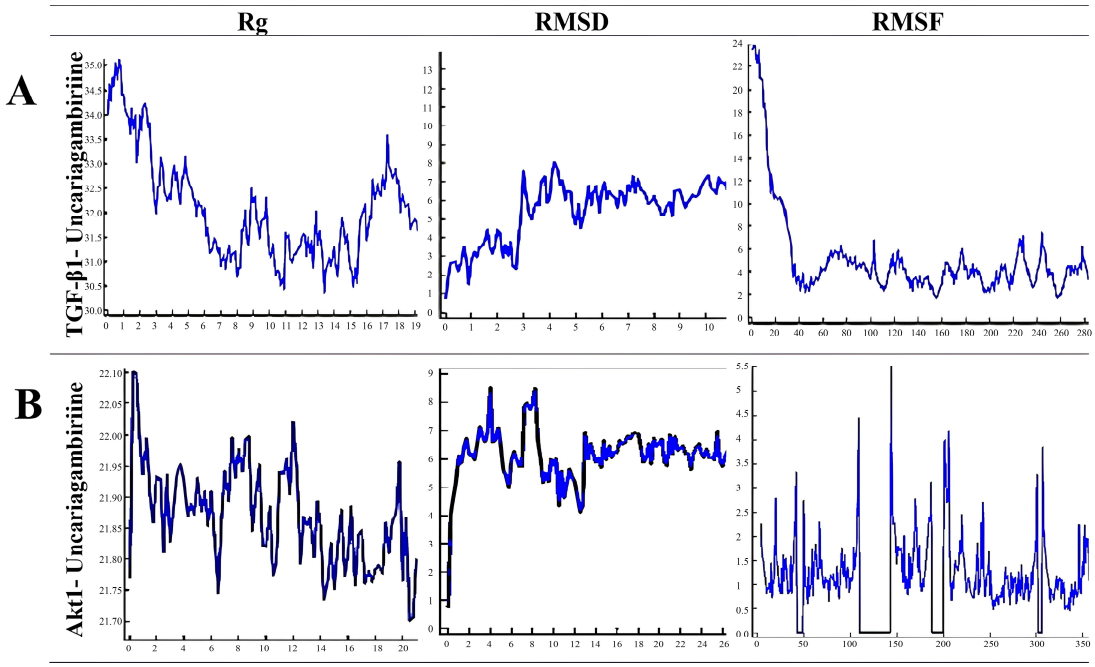


Figure 3. Molecular dynamics simulation of uncariagambiriine with TGFβ1 (**A**) and AKT1 (**B**). Radius of gyration of the solute (Rg), root mean square deviation (RMSD) of protein, and root mean square fluctuation (RMSF) of amino acid residues.

3.3. Cytotoxic and Bioactivity Effect of Gambir Extract and Fraction Towards Keloid Fibroblast

Both bioactivity and toxicity effects are represented as IC_{50} and SI values in Table 4. The lower IC_{50} value indicates the higher effectiveness of the treatment in inhibiting half biological activity of keloid fibroblasts [25]. The ethanol fraction possessed the lowest IC_{50} value, 128.76 ± 0.24 µg/mL, compared with the other samples. It means that the ethanol fraction is more effective at a lower concentration to achieve the same level of action. In addition, gambir extract and fraction possessed an SI higher than 2, indicating high selectivity to keloid fibroblasts. Interestingly, the ethanol fraction had the highest desirable selectivity as well. Therefore, the IC_{50} value of the ethanol fraction was employed to be the reference for the dose of treatments. The treatments were based on $\frac{1}{2}\times$ IC_{50} (64.38 µg/mL), 1× IC_{50} (128.76 µg/mL), and 2× IC_{50} (257.52 µg/mL) values.

Table 4. The effect of Gambir extract and fraction on keloid fibroblast viability and the IC_{50} value of concentration.

Sample	IC_{50} KF	IC_{50} NF	SI ($IC_{50\ KF}/_{NF}$)
Crude extract	249.56 ± 0.22	1096.42 ± 0.52	4.39
Ethanol fraction	128.76 ± 0.24	780.58 ± 0.19	6.06
Ethyl acetate fraction	238.79 ± 0.23	1012.03 ± 0.48	4.24
Hexane fraction	162.07 ± 0.26	570.23 ± 0.49	3.52

SI: selectivity index, KF: keloid fibroblast, NF: normal fibroblast.

3.4. Ex Vivo Analysis of Gambir Ethanol Fraction Effect Towards Keloid Fibroblast

This current study also demonstrated that the gambir ethanol fraction possesses significant inhibitor effects, as evidenced by our ex vivo analysis. The administration of gambir ethanol fraction significantly reduced fibroblast proliferation and migration around the edge of the explant, along with the increase in concentration. The inhibition observed in our ex vivo model suggests a potential mechanism of gambir against keloid disease (Figure 4).

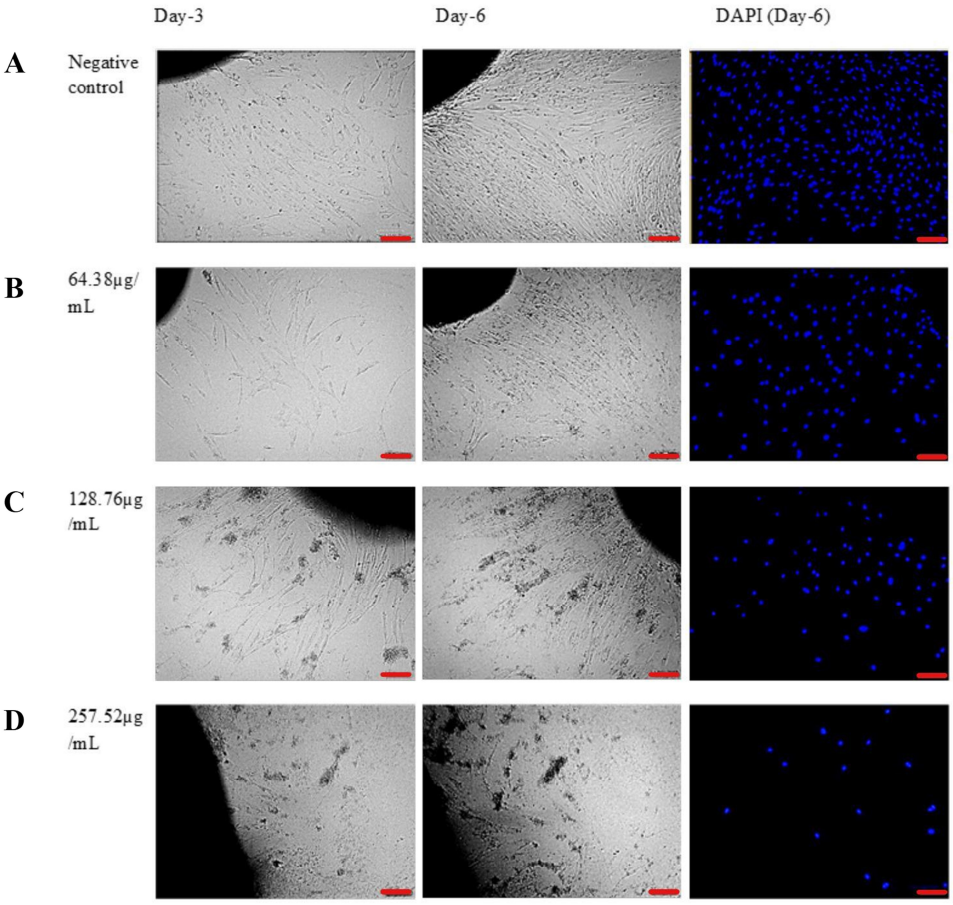


Figure 4. *Cont.*

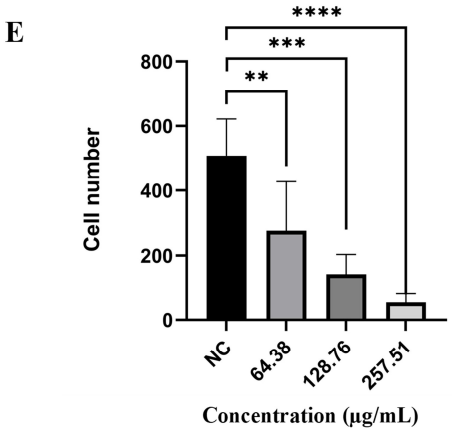


Figure 4. The inhibitory effect of gambir ethanol fraction towards keloid fibroblast migration and proliferation (ex vivo) on day-3 and day-6 after treatments (**A**–**D**) and Semi-quantitative analysis (**E**). DAPI staining was conducted at the end of day-6. The images were captured under an inverted fluorescence microscope at 100× magnification (scale bar 100 µM). Complete media was used as the negative control (NC), and the treatments were 64.38, 128.76, and 257.52 µg/mL of Gambir ethanol fraction. Data was represented as the means ± SD, n = 4. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

4. Discussion

Keloids are pathological scars characterized by an excessive aggregation of fibroblasts and collagen within the reticular dermis [26]. Current therapeutic approaches for keloids, such as corticosteroid injections, surgical excision, and radiation therapy, frequently yield unsatisfactory outcomes and significant side effects. Consequently, there is an urgent need for safer and more effective therapeutic strategies [27]. In this context, herbal-based therapies, which have shown promise in controlling fibrotic diseases, warrant further investigation as a prospective keloid alternative therapy. This study explores the antifibrotic potential of *Uncaria gambir* (W.Hunter) Roxb. (gambir) as an anti-keloid agent. This research identified typical bioactive compounds from gambir extract dan fractions, as summarized in Table 2. Some of them were classified into flavonoids (procyanidin B1, gambiriin A1, and gambiriin A2) and alkaloids (dihydrogambirtannine, uncariagambiriine, and oxogambirtannine) [8]. Notably, the majority of these compounds were detected in the ethanol and ethyl acetate fractions, consistent with the high solubility of both flavonoid and alkaloid compounds in polar and semi-polar solvents, such as ethanol and ethyl acetate. However, the presence of phenolic compounds in the hexane fraction might be due to the limited solvent selectivity of the liquid–liquid fractionation method, which can cause overlapping of compounds across the fractions [28].

Drug–protein network analysis identified nine critical proteins related to keloid pathogenesis that interact with bioactive compounds from gambir, they were AKT1, STAT3, TGFB1, VEGFA, EGFR, IGFR, TIMP, MMP1, and PDGFA. Enrichment analysis indicates that gambir modulates pathways associated with cell proliferation, migration, protein kinase B, and the MAP signaling pathway. These findings are consistent with the hallmark features of keloids, including enhanced fibroblast proliferation and migration driven by dysregulated cytokines and growth factors [29]. These observations align with previous research indicating gambir's bioactive compounds target profibrotic inflammatory cytokines and growth factors to regulate cellular proliferation, migration, and signal transduction activity [15]. Most of the growth factors' signaling activity is primarily mediated by the homodimerization of receptor kinase proteins, including the TGFβ1 signaling pathway [30]. The TGFβ1 signaling pathway plays a central role in keloid pathogenesis through both canonical (via Smad) and noncanonical (non-Smad) mechanisms. Protein kinase B (Akt) is one of the key components of the downstream signaling cascades in the noncanonical

pathway [31,32]. Regarding this, AKT1 and TGFβ1 were selected as target proteins for further analysis using molecular docking and dynamic simulation.

Uncariagambirine exhibited the strongest binding affinity to AKT1 and TGFβ1. The binding energy between protein and ligand represents the strength of interaction. The smaller the binding energy, the stronger the interaction [22]. The molecular dynamics simulation was conducted to evaluate the stability of the compound protein in fluid conditions resembling the human body environment [33]. The result suggests stable solute behavior, dynamic ligand flexibility, and localized solute fluctuations that influence ligand-receptor interactions. Further studies, such as free energy calculations, could clarify the binding mechanisms and stability of the complex.

The cytotoxic and bioactivity effects of gambir extract and its fraction towards keloid fibroblasts validate the antifibrotic potential of gambir. Among the tested fractions, the ethanol fraction exhibited the highest abundance of bioactive compounds and the strongest cytotoxic effect on keloid fibroblasts, with an IC_{50} value of 128.76 ± 0.24 μg/mL. In addition, the ethanol fraction showed the highest selectivity index against the keloid fibroblast, indicating the low cytotoxicity of the normal fibroblast [34]. Ex vivo analysis further highlighted the potential of the ethanolic fraction of gambir against keloid. Treatment with the ethanol fraction significantly inhibited fibroblast proliferation and migration in keloid explants (Figure 4). The inhibition of fibroblast migration is particularly noteworthy, as it suggests that gambir not only reduces existing fibroblast activity but may also prevent the spread of pathological fibroblasts to surrounding tissues. Further experimental studies are required to investigate the mechanisms of action of gambir bioactive compounds in the keloid pathomechanism, such as using keloid-derived fibroblasts for an in vitro approach.

This study reveals the promising potential of gambir's ethanol fraction as a novel antifibrotic candidate, with uncariagambiriine identified as the primary bioactive compound. The combined in silico and experimental results demonstrated that gambir bioactive compounds (procyanidin B1, gambiriin A1, dihydrogambirtannine, uncariagambiriine, and oxogambirtannine) may have significant potential in inhibiting fibroblast proliferation and migration. These findings emphasize Gambir's ability to modulate keloid cell proliferation, migration, and extracellular matrix synthesis via multiple targets and pathways associated with keloid pathogenesis [35]. Understanding the underlying mechanism of these effects is essential. Therefore, further research is required to elucidate the pathway mechanism of gambir in keloid, including analysis of the key protein expression involved in keloid pathogenesis. Additionally, the low selectivity partition of liquid–liquid fractionation is acknowledged as the limitation of this study. Therefore, further separation using more effective techniques such as column chromatography is recommended for future studies. Exploring the synergistic effects of gambir's bioactive compounds with existing keloid therapies could enhance its clinical applicability. Hence, further experimental study needs to be conducted for future research to enhance the potential of gambir as an effective anti-keloid agent.

5. Conclusions

In conclusion, procyanidin B1, gambiriin A1, dihydrogambirtannine, uncariagambiriine, and oxogambirtannin have been identified as the primary typical bioactive compounds in Gambir with potential antikeloid properties. Uncariagambiriine demonstrated the most potent compound, characterized by its high binding affinity to key target proteins, AKT1 and TGFβ1. The ethanol fraction exhibited the lowest IC50 value (128.76 ± 0.24 μg/mL) and highest SI (6.32) to keloid fibroblasts, highlighting its superior potential as an antikeloid candidate. Ex vivo analysis further confirmed that the ethanolic fraction of Gambir significantly inhibited keloid fibroblast proliferation and migration. These findings underscore

the potential of the Gambir ethanolic fraction as a promising antifibrotic agent for keloid treatment, warranting further investigation and development for clinical applications.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/biologics5030018/s1, Table S1: LC-MS analysis of Gambir extract and fraction.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Faculty of Medicine, Universitas Indonesia—Cipto Mangunkusumo Hospital (No: KET-1206/UN.F1/ETIK/PPM.00.02/2023, approval date: 21 September 2023).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data were available in this manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest in this study.

Abbreviations

The following abbreviations are used in this manuscript:

LCMS/MS	liquid chromatography–mass spectrometry
ECM	extracellular matrix
TGFβ1	transforming growth factor beta 1
AKT1	AKT serine/threonine kinase 1
MMP1	matrix metallopeptidase 1
UPLC	ultra-performance liquid chromatography
ESI	electrospray ionization
SMILE	canonical simplified molecular-input line-entry
DAVID	database for annotation, visualization, and integrated discovery
PDB	protein data bank
Rg	radius of gyration
RMSD	root mean square deviation
RMSF	root mean square fluctuation
PBS	phosphate-buffered saline
BSC	biosafety cabinet
DAPI	4′,6-diamidino-2-phenylindole
GO	gene ontology
BP	biological processes
MF	molecular functions
CC	cellular components
SI	selectivity index

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