

In Vitro Assay and Study Interaction of *Uncaria gambir* (Hunter) Roxb. as Anti-fibrotic Activity Against A549 Cell Line

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ABSTRACT

Aim: The aim of this study is to finding inhibitor potential from several compounds in gambir plant by using *in vitro* MTT assay and study interaction with molecular docking. The interaction of amino acids on the binding site with substances in the gambir plant was analyzed to determine its potential as a herbal-based therapy candidate for pulmonary fibrosis. **Material and Methods:** Protein target using TGFβ1 and NF-κB and compounds from gambir plant ((+)-Catechin, Epigallocatechin gallate, (+)-Epicatechin, Gambirinin A1, Gambirinin A2, Gambirinin B1, Gambirinin B2, Gambirinin C, Procyanidin B1, Procyanidin B3). **Result:** The results from docking analysis observed that compounds from gambir fruit contain anti-fibrotic activity which act by inhibiting DNA transcription of NF-κB and TGFβ1 receptors. The compound Procyanidin B3, an essential amino acid, contains a hydrogen bond with the greatest NF-κB inhibitory activity on Gly214 and Lys337. Compounds from *Uncaria gambir* (Hunter) Roxb. can be an inhibitor to TGFβ1, all the compounds are on the active site of TGFβ1, and use native ligand which is an inhibitor of TGFβ1 (Naphthyridine). The positive compound catechin has the highest inhibitory activity. Gambirinin B1 and Gambirinin A2 are the most identical compounds with similar affinity binding value. *Uncaria gambir* (Hunter) Roxb. is already a proven anti-fibrotic which is further confirmed by (IC50: 19,255 ± 1.08 µg/ml, p < 0.05) in A549 cell line.

Conclusion: The results demonstrated that Gambirinin have cytotoxic effects and was found potentially as anti-fibrotic by MTT assay and *in silico* evaluation.

Key words: Gambirinin compounds, Inhibitor of p50 NF-κB, Molecular docking, Pulmonary fibrosis, TGFβ1 receptors.

INTRODUCTION

Pulmonary fibrosis is a condition of scar tissue formation that involves infiltration of inflammatory cells, fibroblasts proliferation, reactive oxygen species (ROS), and excessive accumulation of extracellular matrices to the pulmonary parenchymal tissues and can cause dysfunction to pulmonary function.^{1,2} The appropriate treatment for pulmonary fibrosis is still a challenge. The use of chemical substances effective for pulmonary fibrosis treatment does not always provide consistent significant results due to adverse events.³ Several methods to prevent and reduce pulmonary fibrosis have been widely conducted using drugs that interact with TGFβ and have the potential to activate anti-fibrosis. The drugs were tranilast (inhibit the activity and secretion of TGFβ), losartan (TGFβ induction blockade), PPAR-γ agonists (damaging Smad3 signal transduction), pirfenidone (inhibit the production of TGFβ), and halofuginone.³ The effectivity limitations of these chemical substances in overcoming pulmonary fibrosis lead to the utilization of natural ingredients as an alternative.

Anti-fibrotic drugs such as Pyfenidone were developed for the treatment of idiopathic pulmonary fibrosis. Because fibroblast activities in inflammatory conditions have the same

characteristics as cancer-related fibroblasts that actively contribute to phenotype, the search for anti-fibrotic drugs can be through a repurposed approach as an anti-cancer drug.⁴ Furthermore, it has been shown that drug such as pirfenidone can inhibits proliferation, and epithelial-mesenchymal transition of a human epithelial cell line,⁵ disrupted tumor-stromal interactions in pancreatic cancer,⁶ and inhibited TGFβ1-induced overexpression of collagen type I in A549 cells.⁷

TGF-β is involved in pulmonary fibrosis promoting progression by both autocrine and paracrine mechanisms.¹ Studies in adenovirus transfected or transgenic revealed that TGF-β contributed to HSC activation and fibrotic damage, and that blocking TGF-β signalling inhibited the fibrotic process.^{2,3} TGF-β1/Smads signalling is required for fibrosis.⁴

However, there is lack in studies addressing the exact molecular mechanism behind this effect, which is a common challenge in the natural products research. Therefore, molecular docking studies can explain the interactions between phytochemicals and the molecular targets, which could be a successful approach to develop new drugs.

Gambir or *Uncaria gambir* (Hunter) Roxb. is a specific local plant in South East Asia, especially Indonesia and Malaysia. Gambir contains polyphenol substances with its main component of

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flavonoid (+)-catechin comprises of 40-80% dry extract weight, and (-)-epicatechin 1.5%, procyanidin B1, procyanidin B3, and gambirinin each comprises of 1%. The most commonly used chemical substance of gambir is catechin and tannin, which is the polymer form of catechin.^{7,8} Gambir has antioxidant properties, can inhibit the activation of NFκB by suppressing the formation of collagen, TIMP-1 through *in vivo* fibrolysis, and fibropreventive mechanism in the fibrotic. The main component of Gambir is a flavonoid (+)-catechin, which comprises of 40-80% of dried sap and had been proven to have a strong antioxidant activity, almost comparable to vitamin C *in vitro*, and inhibits the activation of NFκB *in vivo*. Thus, enables its role as anti-fibrotic therapy in the lungs and pleura.

Structure-based drug design (SBDD) is a chemical computation method which utilizes the information from target protein structures to determine protein active site that bonds with substances. Based on the active site prediction, the expected substance can be designed to attach with the target protein and provide biological activity. Using the information of target structure and ligand physicochemical property, screening of interaction between known active substances (ligand) on the prediction of protein active site can be performed. Based on the obtained information, this new substance design is expected to be more potent than the available substances. One of the commonly used SBDD methods is molecular docking.⁹

In silico examination there is a term for an experiment or an exam conducted by a computer simulation method. The *in silico* test has become a method used to initiate the discovery of new compounds and to increase efficiency in optimizing the activity of parent compounds. The purpose of *in silico* exams is to predict, give hypotheses, make new discoveries or new advances in medicine and therapy. One of the *in silico* tests was carried out by the molecular docking method of a prospective compound of a drug compound with the selected receptor. Docking is an attempt to align between ligands which are small molecules into receptors which are large protein molecules, by paying attention to the properties of both.¹⁰ This study conducted *in silico* testing of the molecular docking method of binding affinity results of molecular docking and the interaction of amino acids on the binding site with substances in the gambir plant was analyzed to determine its potential as a herbal-based therapy candidate for pulmonary fibrosis.

MATERIALS AND METHODS

Computational methods

All computational studies to perform the *in silico* experiments were carried out using MacBook Air with operating system Mac OS High Sierra 10.13.3, processor Intel Core i5 1.8GHz, RAM 8 GB, 1600 MHz DDR3. Marvin Sketch to draw ligand structure in 2 and 3 dimensions. AutoDockVina for docking process. AutoDockTools for docking result analysis and 3D visualization of the ligand-receptor complex. LigPlus for analysis of residual amino acids of the ligand-receptor complex in 2D.

Druglikeness

DruLiTo is open source software to can calculate different molecular properties and screen the molecules based on drug likeness rules using 'The Lipinski rule of five'.

Protein preparation

Structures of protein targets (+)-Catechin, Epigallocatechin gallate, (+)-Epicatechin, Gambirinin A1, Gambirinin A2, Gambirinin B1, Gambirinin B2, Gambirinin C, Procyanidin B1, Procyanidin B3.

Receptor preparation

Download receptor from www.rcsb.org in .pdb format. Separate chain receptor and ligand. Eliminate water using delete water in Autodock software. Add hydrogen atom (polar only) and merge non-polar. Add Gasteiger charges on the receptor. Save file in .pdbqt.

Ligand preparation

Separate the original PDB ligand from a receptor-ligand complex (specific to ligands from receptor-ligand PDB complex). Draw nine substances in .pdb format using Marvin Sketch.

Docking method validation

Conduct docking on PDB ligand using the optimization of grid box size and center grid position. Choose the optimal size of the grid box and grid center by determining the result of docking.

Docking and docking result analysis

Make 1 folder containing protein and ligand in.pdbqt format. Configure docking (protein file name, ligand file name, grid box coordinate, grid box size, output file name, and model numbers produced) Implement docking using AutodockVina through the Terminal. The best docking model from docking file output (.pdbqt) was separated using vina_split. Analysis and visualization of the ligand-receptor complex through Ligplus.

Preparation of extract

The fresh fruits of *Uncaria gambir* (Hunter) Roxb were washed under running water and shade dried. Finely powdered by mechanical grinder and extracted with 90% ethanol by maseration maintaining at room temperature 3 x 24 hours. Evaporated the solvent by rotary evaporator and brownish gummy exudates were obtained. The crude *Uncaria gambir* (Hunter) Roxb extract was used for citotoxicity properties. The fraction yield of extract *Uncaria gambir* (Hunter) Roxb was calculated by using the formula.

$$\% \text{ yield} = \frac{\text{weight of crude extract}}{\text{weight of raw material}} \times 100$$

The fraction yield of ethanolic extract of *Uncaria gambir* (Hunter) Roxb was found to be 7.15% w/w.

Total phenol content

The estimation of the total phenol content was done by using folin ciocalteu reagent method. 2.5ml of saturated Na₂CO₃ was added to the pre incubated 0.5ml of EOEt extract with 0.1ml of folin ciocalteu reagent (0.5N) for 15min in optimum temperature. Measure the absorbance at 760nm using quercetin as standard. The total phenol content was uttered as standard equivalent (mg/g).

Total flavonoid content

The total flavonoid content was estimated by using Aluminum chloride method. The mixture (3.0 ml) contains 1.0 ml extract of *Uncaria gambir*, 0.5ml of aluminum chloride (1.2%) and 0.5 ml of 120 mM potassium acetate were pre-incubated in room temperature for 30 min and at 415 nm the absorbance was measured using catechin as standard. The total flavonoid content was uttered in terms of standard equivalent (mg/g).

MTT assay

Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS; DMEM and RPMI1640 are also alternatives that work well. Aspirate and add fresh culture medium every 2-3 days. A549 cell doubling time is 48 hours. To passage cells, rinse cell monolayer with 1x

PBS twice and add pre-warmed (37°C) 0.05% Trypsin-EDTA solution to cover the bottom of the flask; incubate for 5 – 7 minutes. As cells detach, neutralize the Trypsin by adding 4x volume of complete growth medium with 10% FBS and gently resuspend the cells by pipetting

To avoid clumping do not agitate the cells by shaking the flask while waiting for detachment. Split cells 1:4 every 3 days or 1:8 every 6 days. Cultures should be incubated at 37°C in a humidified atmosphere with 5% CO₂. If cells are difficult to detach the suggestions are cell-to-cell junctions are tight due to cell growth being 100% confluent and dissociation agent cannot reach cell interface; subculture cells before confluent. Use higher concentration of dissociation agent; incubate flask at 37°C to increase enzymatic activity. Wash flask twice with sterile 1x PBS prior to addition of the dissociation agent.

RESULTS AND DISCUSSION

In silico pharmacokinetic properties and toxicities were predicted using ADME property explorer using DruLiTo is an open source virtual screening tool. This calculation is based on the various drug-likeness rules like Lipinski's rule. Drug-likeness rules like Lipinski's rule were predicted of chemical constituents from extract of gambir analysis by online software at http://www.niper.gov.in/pi_dev_tools/DruLiToWeb/DruLiTo_index.html. Results of ADME analysis are shown in Table 1.

Drug likeness can be estimated for any molecule, and does not evaluate the actual specific effect that the drug achieves (biological activity). Simple rules are not always accurate and may unnecessarily limit the chemical space to search: many best-selling drugs have features that cause them to score low on various drug-likeness indices. It is estimated from the molecular structure before the substance is even synthesized and tested. A druglike molecule has properties such as Solubility in both water and fat, Potency at the biological target, and Ligand efficiency and lipophilic efficiency.

Docking is an attempt to align between ligands which are small molecules into receptors which are large protein molecules, by paying

attention to the properties of both. Result of docking approach with ligand from gambir extract with NF-κB p50 and TGF-β1:

Substance docking with NF-κB p50

All substances studied were tethered to the binding site of the receptor (ID PDB: 1SVC) using AutodockVina. The grids were prepared for each protein with a similar center and border-box size set at 0.375 Å distance, with a grid box size of 60 x 60 x 60. The grid center coordinate was (x = 37.569; y = 24.716; z = 38.430) (Figure 1).

NF-κB is the regulation center of the stress response, activated by various stress conditions, including physical and oxidative stress. From the docking results, the substances acted in DNA transcription inhibition of NF-κB. Based on molecular docking results, three compounds from gambir plant: gambiriin A1, procyanidin B1 and procyanidin B3 have the best binding affinity score -8.4; -8.4; and -8.6 kcal/mol (Table 2).

Procyanidin B3 had a hydrogen bond with the highest inhibitory activity (Figure 2). Residuals interacted with Procyanidin B3, Procyanidin B1 and Gambiriin A1 ligands were in the IPT domain of NF-κB transcription factor (251-352), GRR area (374-396), and interaction domain of CFLAR/CASP8 and FADD-like apoptosis regulator (437-970). These three ligands had similar amino acid bonding on Gly214 and Lys337. The three ligands in successive order were Procyanidin B3, Procyanidin B1, and Gambiriin A1 had 9, 11, and 12 interactions with amino acid residue (Table 3). This showed that even though Procyanidin B3 had the fewest amino acid interaction, the interacting amino acid was an essential amino acid important to determine the binding affinity. The interaction of substances in the Gambir plant is expected to inhibit the transcription of NF-κB.

Docking of substance with TGF-β1

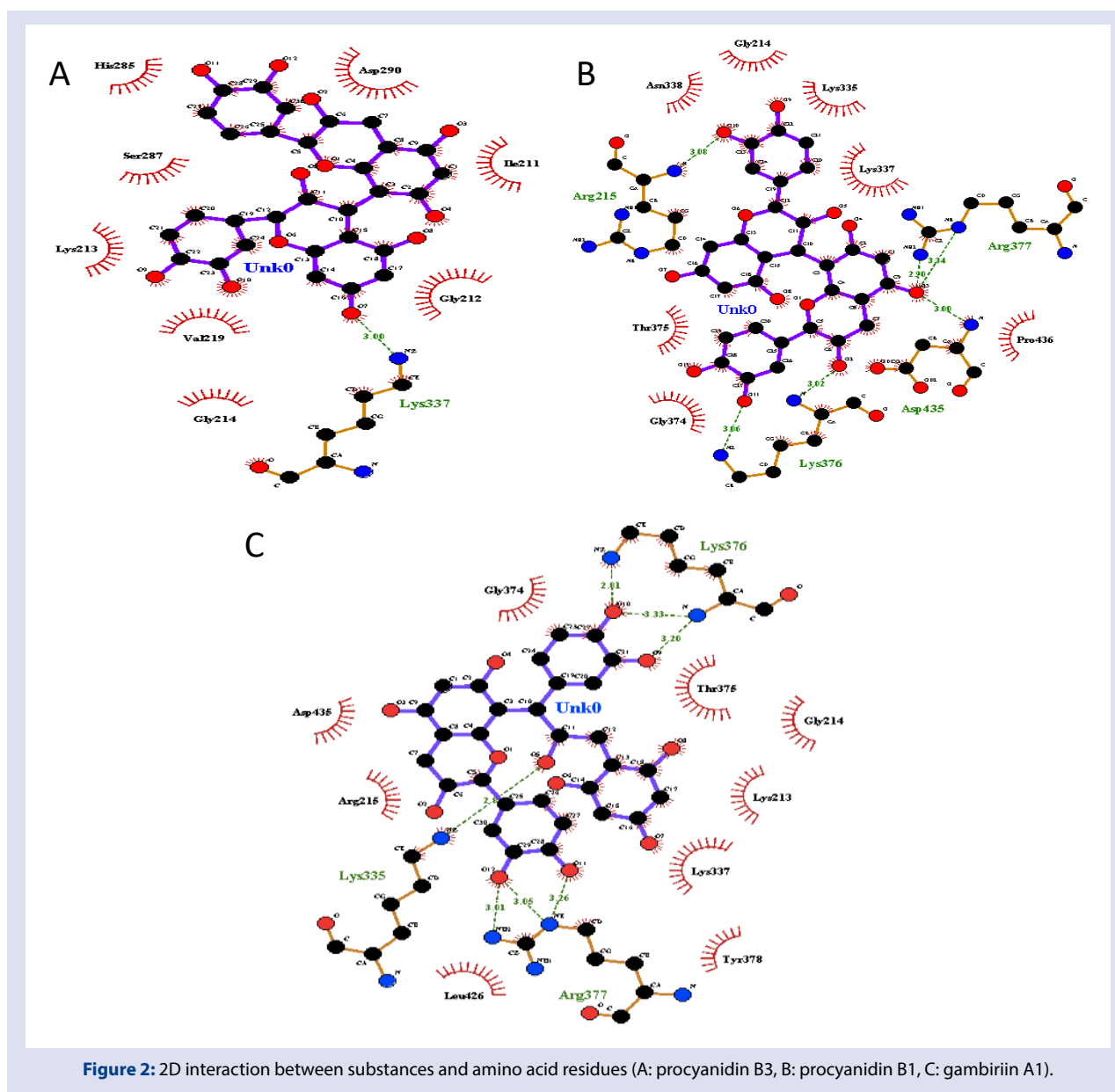
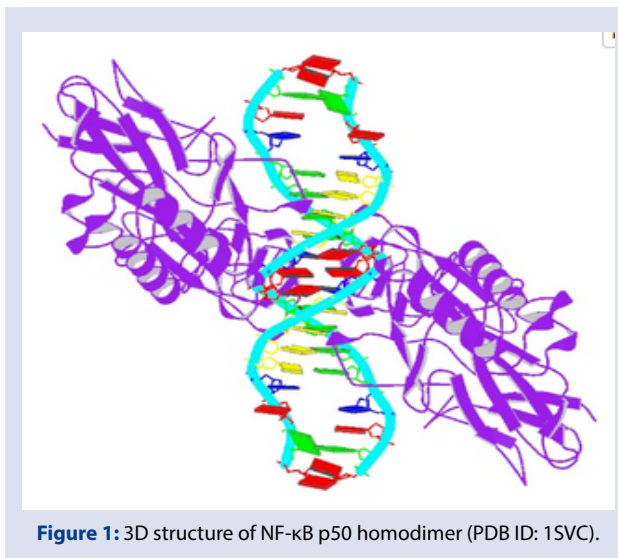
All substances were attached to the receptor binding site (PDB ID: 1VJY) using AutodockVina. The grids were prepared for each protein with a similar center and border-box size determined at 0.375 Å

Table 1: Druglikeness Lipinski's rule of chemical constituents of *Uncaria gambir* (Hunter) Roxb.

Compounds name	Molecular Weight g/mol	TPSA A ²	LogP	Lipinski
(+)-Catechin	322.74	86.99	1.70	Y
Epigallocatechin gallate	299.32	81.78	2.33	Y
(+)-Epicatechin	285.29	92.78	1.64	Y
Gambiriin A1	316.31	96.22	2.66	Y
Gambiriin A2	368.77	116.45	2.33	Y
Gambiriin B1	344.36	85.22	3.20	Y
Gambiriin B2	562.53	200.52	3.44	Y
Gambiriin C	562.53	200.52	3.07	Y
Procyanidin B1	578.53	220.75	2.58	Y
Procyanidin B3	578.53	220.75	2.58	Y

Table 2: Binding affinity score from docking gambir compounds with p50 NF-κB.

No	Compounds	ΔG (kcal/mol)
1	(+)-Catechin	-7.0
2	Epigallocatechin gallate	-7.2
3	(+)-Epicatechin	-6.5
4	Gambiriin A1	-8.4
5	Gambiriin A2	-8.4
6	Gambiriin B1	-7.8
7	Gambiriin B2	-8.3
8	Gambiriin C	-8.2
9	Procyanidin B1	-8.4
10	Procyanidin B3	-8.6



distance with a grid box size of 40 x 40 x 40. The grid center coordinate was (x = 14.129; y = 67.092; z = 5.249) (Figure 3).

From the results of the docking, the substances in the Gambir plant can be TGF β R1 inhibitors. Three compounds from gambir such as catechin, epigallocatechin gallate and procyanidin B3 showed best binding affinity score -9.4; -9.0 and -9.3 kcal/mol (Table 4).

From the analysis result of ligand-receptor, all substances were in the active site of TGFBR1 (211..215, 219, 230, 232, 260, 280, 283, 287, 289, 333, 335, 337, 338, 340, 351, 354, 374..377), as well as the native ligand, which was a TGFBR-1 inhibitor (Naphthyridine). The high inhibitory activity of (+)-catechin can be caused by interaction similarity between native ligand, which was not seen in other substances, i.e. at 230, 232, 260, 280, 340, 351. The amino acid residual interactions of (+)-catechin, Procyanidin B3, and (+)-Epigallocatechin gallate were 13, 9, and 12, respectively (Table 5). Seen from the amino acid interaction mapping between (+)-catechin ligand with the native ligand, the binding affinity value was inferior to native ligand due to several interactions that had no hydrogen bond (Figure 4).

MTT assay

Viability and Characterization of Cell lines A549 lung fibrosis used in the MTT test. The cytotoxicity activity of the ethanolic extract of *Uncaria gambir* (Hunter) Roxb. was carried out by using MTT assay with different concentration on A459 cell lines. Results of anticancer activity on A459 are shown in Table 6.

Antifibrosis inhibitors and agents, commonly fibroblasts in inflammatory conditions agents are used in management of tumor growth factors (e.g., vascular endothelial-derived growth factor, transforming growth factor beta (TGF β), hepatocyte growth factor, epidermal growth factor, and fibroblast growth factor. The ethanolic extract of *Uncaria gambir* (Hunter) Roxb. shows the concentration dependent inhibition. The ethanolic extract of *Uncaria gambir* (Hunter) Roxb was found to have strong inhibitory activity (IC₅₀ 19,255 \pm 1.08 μ g/ml) while, considering the standard drug cisplatin (1,02121E-06 \pm 0.03 μ g/ml) (Figure 5) with R 0,9178 and 0,9373 respectively. In consequence, the IC₅₀ of ethanolic extract of *Uncaria gambir* (Hunter) Roxb extracts might be due to the presence of phenols, phenolic total.

Table 3: Interaction analysis by mapping amino acid residues.

Amino acid	Procyanidin B3	Procyanidin B1	Gambiriin A1
Ile211	√	-	-
Gly212	√	-	-
Lys213	√	-	√
Gly214	√	√	√
Arg215	-	√*	√
Val219	√	-	-
His285	√	-	-
Ser287	√	-	-
Asp290	√	-	-
Lys335	-	√	√*
Lys337	√*	√	√
Asn338	-	√	-
Gly374	-	√	√
Thr375	-	√	√
Lys376	-	√*	√*
Arg377	-	√*	√*
Tyr378	-	-	√
Leu426	-	-	√
Asp435	-	√*	√
Pro436	-	√	-
Total interaction	9	11	12

Residual analysis (*: hydrogen bond)

Table 4: Binding affinity score from docking gambir compounds with TGF- β 1.

No	Compounds	Δ G (kcal/mol)
1	(+)-Catechin	-9.4
2	Epigallocatechin gallate	-9.0
3	(+)-Epicatechin	-8.6
4	Gambiriin A1	-8.4
5	Gambiriin A2	-8.0
6	Gambiriin B1	-8.4
7	Gambiriin B2	-8.2
8	Gambiriin C	-8.5
9	Procyanidin B1	-9.0
10	Procyanidin B3	-9.3
11	Native ligand	-9.7

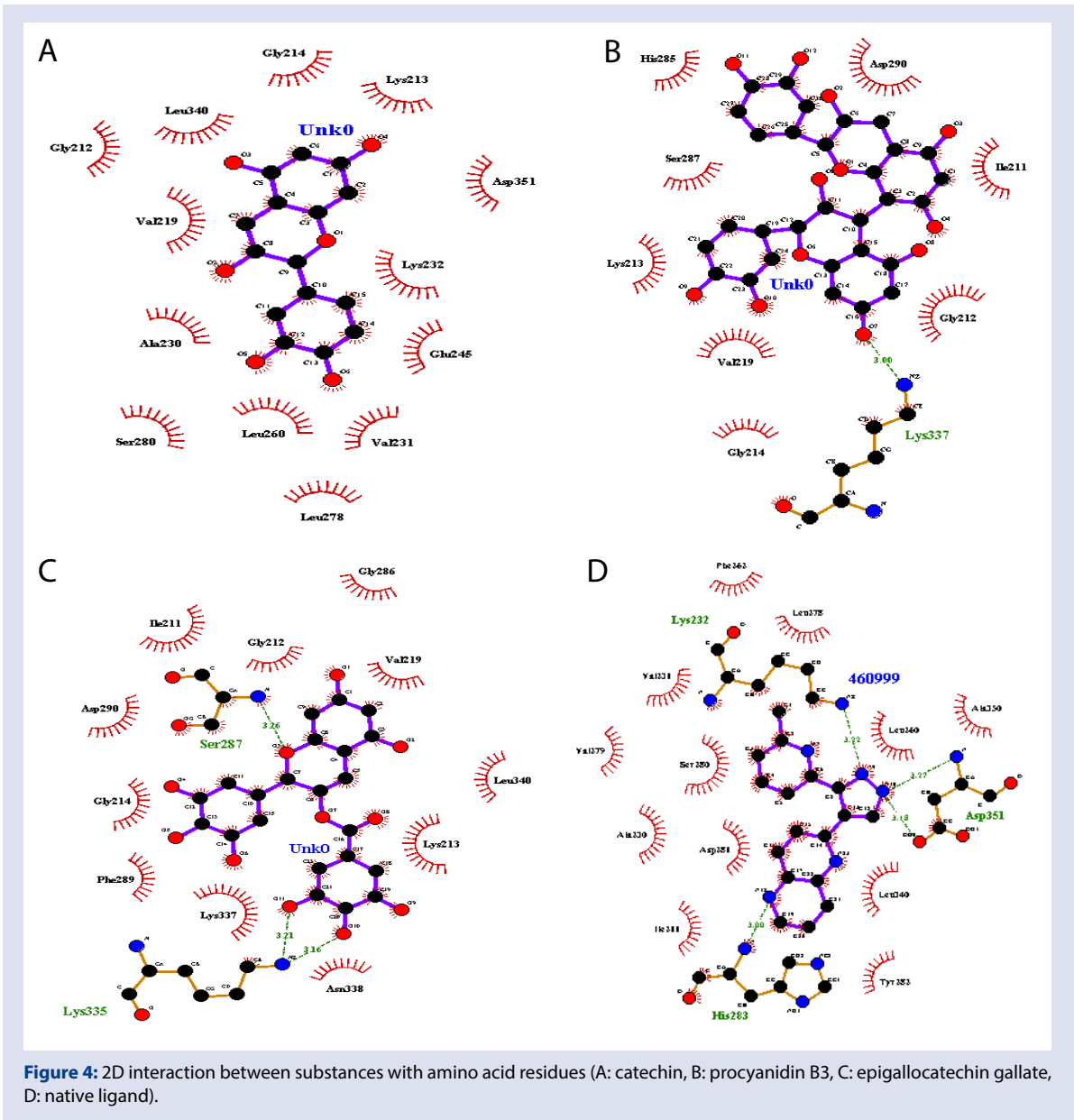
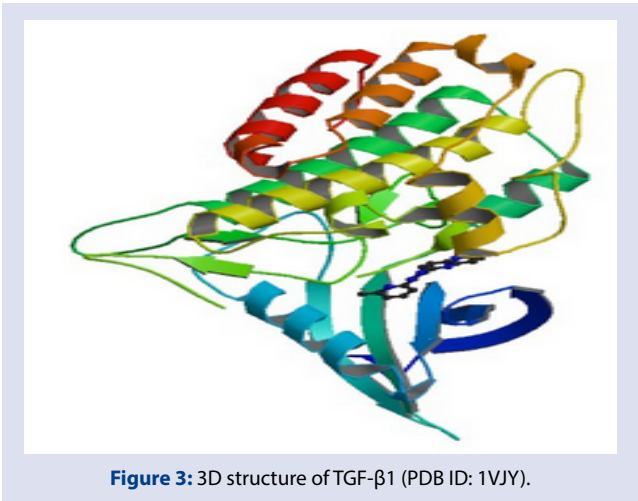


Table 5: Binding affinity score from docking gambir compounds with TIMP-1.

No	Compounds	ΔG (kcal/mol)
1	(+)-Catechin	-8.4
2	Epigallocatechin gallate	-9.5
3	(+)-Epicatechin	-7.3
4	Gambiriin A1	-8.4
5	Gambiriin A2	-9.7
6	Gambiriin B1	-10.0
7	Gambiriin B2	-9.7
8	Gambiriin C	-9.5
9	Procyanidin B1	-9.4
10	Procyanidin B3	-9.2

Table 6: IC₅₀ value of ethanolic extract of *Uncaria gambir* (Hunter) Roxb with A549 cell line.

Concentration	Log concentration	Absorbance	ic50	
			Standart Cisplatin	ethanolic extract of <i>Uncaria gambir</i>
1,5625	0,19382002	0,159	77,69985975	0,459
3,125	0,49485002	0,145	79,61664329	0,624
6,25	0,79588001	0,125	82,42169238	0,355
12,5	1,09691001	0,121	82,9827022	0,321
50	1,69897000	0,100	86,02150538	0,266
100	2	0,101	85,8345021	0,133
200	2,30102999	0,121	-	0,125
	Control	0,713		0,713

IC₅₀ value less than 100 is considered as an active extract with anticancer activity. As shown in Table 6, ethanolic extract of *Uncaria gambir* have IC₅₀ value lower than 100 µg/mL, that is assigned as active extract. ethanol extracts showed strong anticancer activity. Consistent and agree with the computational *in silico* molecular docking the substances in the Gambir plant can be TGFβ1 inhibitors. Three compounds from gambir such as catechin, epigallocatechin gallate and procyanidin B3 showed best binding affinity score -9.4; -9.0 and -9.3 kcal/mol respectively and comper with IC₅₀ value of 19,255 ± 1.08 µg/ml. This result indicates that ethanol extract of *Uncaria gambir* potential to be developed as an anti fibrotic drug assessment, ethanol extract of *Uncaria gambir* presence of polyphenols (56.4 + 6.47 mg eq. QE/g Ext), demonstrated the greater anticancer activity than the other extract on A549 lung cancer cells.

CONCLUSION

Molecular docking simulation of gambir compounds to discover its anti-fibrotic activity has been done to explore its potential for inhibiting DNA transcription of NF-κB, TGF-β1 receptors. Three compounds from gambir plant such as catechin, epigallocatechin gallate and procyanidin B3 showed best binding affinity score -9.4; -9.0 and -9.3 kcal/mol respectively and comper with IC₅₀ value of 19,255 ± 1.08 µg/ml. This result indicates that ethanol extract of *Uncaria gambir* potential to be developed as an anti-fibrotic drug assessment, ethanol extract of *Uncaria gambir* presence of polyphenols (56.4 + 6.47 mg eq. QE/g Ext), demonstrated the greater anticancer activity than the other extract on A549 lung cancer cells.

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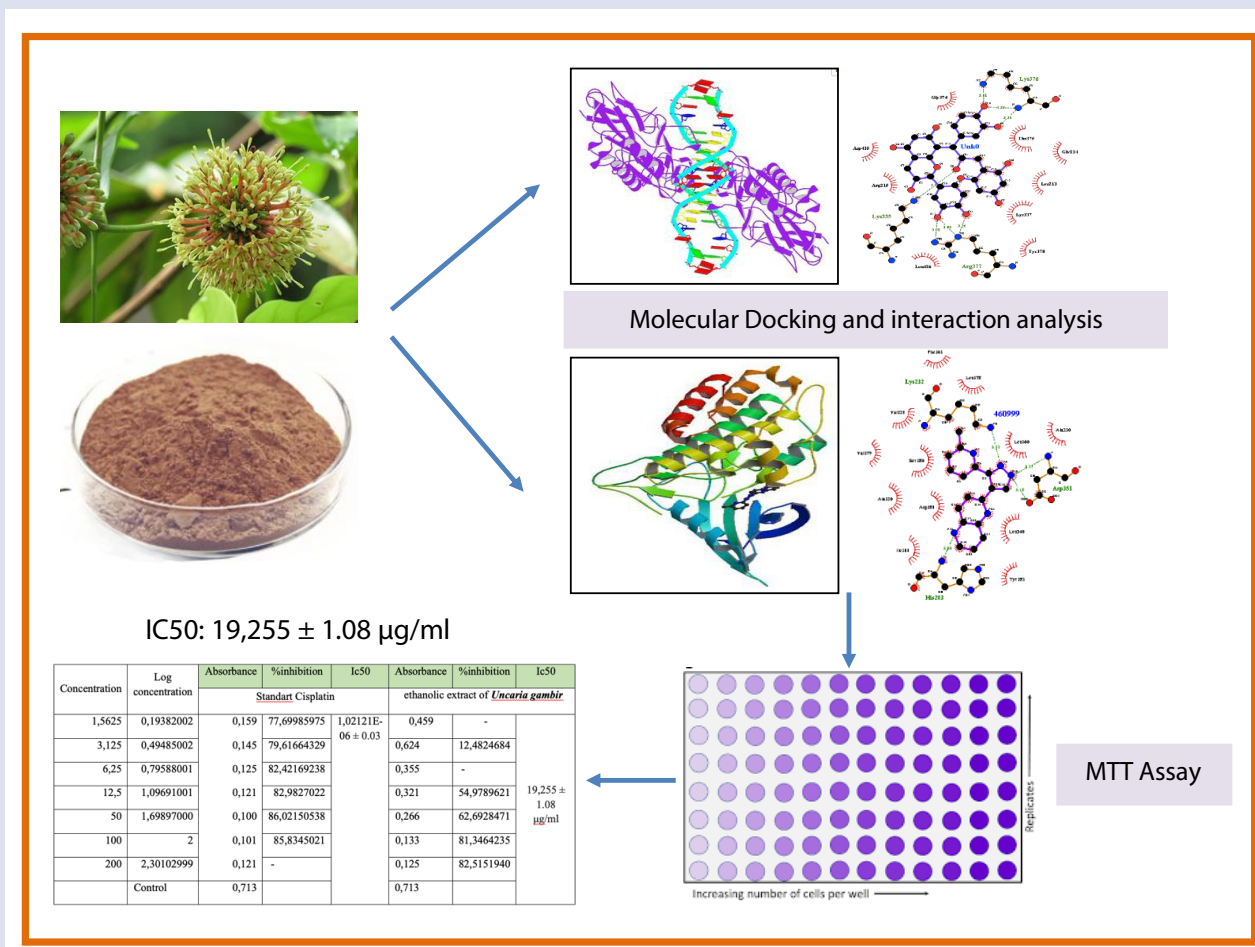
CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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GRAPHICAL ABSTRACT



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