



# Predicting activities of *Clinacanthus nutans* (Burm.f.) Lindau metabolites against molecular targets in metabolic dysfunction-associated steatotic liver disease

[Predicción de la actividad de los metabolitos de *Clinacanthus nutans* (Burm.f.) Lindau frente a dianas moleculares en la enfermedad hepática esteatósica asociada a disfunción metabólica]

Nurina Hasanatuludhhiyah<sup>1,2,3</sup>, Arifa Mustika<sup>2\*</sup>, Viskasari P. Kalanjati<sup>2</sup>, Kautsar U. Haq<sup>4,5</sup>,  
Muhammad Miftahussurur<sup>6,7</sup>, Hirokazu Wakuda<sup>3</sup>, Naoto Uemura<sup>3</sup>

<sup>1</sup>Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga Surabaya, Indonesia.

<sup>2</sup>Department of Anatomy, Histology, and Pharmacology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

<sup>3</sup>Department of Clinical Pharmacology and Therapeutics, Faculty of Medicine, Oita University, Yufu, Oita, Japan.

<sup>4</sup>Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia.

<sup>5</sup>Research Center for Bio-Molecule Engineering (BIOME), Universitas Airlangga, Surabaya, Indonesia.

<sup>6</sup>Division of Gastroentero-Hepatology, Department of Internal Medicine, Faculty of Medicine – Dr. Soetomo Teaching Hospital, Universitas Airlangga, Surabaya, Indonesia.

<sup>7</sup>Helicobacter pylori and Microbiota Study Group, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.

\*E-mail: [arifa-m@fk.unair.ac.id](mailto:arifa-m@fk.unair.ac.id)

## Abstract

**Context:** Metabolic dysfunction-associated steatotic liver disease (MASLD) is the most prevalent chronic liver disorder worldwide, yet pharmacological treatment options remain limited. Therapeutic agents that modulate both metabolic and hepatic pathological pathways are urgently needed.

**Aims:** To identify chemical constituents from the ethanolic extract of *Clinacanthus nutans* and predict their interactions with validated molecular targets implicated in MASLD using *in silico* methodologies.

**Methods:** Metabolite profiling was conducted using ultra-high-performance liquid chromatography–mass spectrometry (UHPLC–MS). Identified compounds were evaluated for drug-likeness based on Lipinski, Ghose, and Veber criteria. Compounds meeting these criteria were subjected to ADMET profiling and molecular docking against 20 MASLD-associated targets using validated docking protocols. The potential compound underwent molecular dynamics analysis.

**Results:** Seventeen compounds were identified from the *C. nutans* extract. Six metabolites, along with stearidonic acid, demonstrated favorable pharmacokinetic properties. Among these, a thiophene-based benzoic acid derivative and tagalsin Q exhibited strong binding affinities toward CCR2, ChREBP, and SGLT2, with interaction profiles comparable to native ligands.

**Conclusions:** Metabolites of *C. nutans* possess predicted bioactivity against CCR2, ChREBP, and SGLT2, supporting their potential utility as multi-target phytotherapeutic agents for MASLD.

**Keywords:** *Acanthaceae*; ADMET prediction; *Clinacanthus nutans*; hepatitis; molecular docking; natural compounds.

## Resumen

**Contexto:** La enfermedad hepática esteatósica asociada a disfunción metabólica (MASLD) es el trastorno hepático crónico más prevalente en todo el mundo, pero las opciones de tratamiento farmacológico siguen siendo limitadas. Se necesitan urgentemente agentes terapéuticos que modulen tanto las vías metabólicas como las patológicas hepáticas.

**Objetivos:** Identificar los componentes químicos del extracto etanólico de *Clinacanthus nutans* y predecir sus interacciones con dianas moleculares validadas implicadas en la MASLD utilizando metodologías *in silico*.

**Métodos:** Se realizó un perfil metabólico mediante cromatografía líquida de ultra alto rendimiento y espectrometría de masas (UHPLC-MS). Se evaluó la similitud farmacológica de los compuestos identificados según los criterios de Lipinski, Ghose y Veber. Los compuestos que cumplían estos criterios se sometieron a un perfil ADMET y a un acoplamiento molecular con 20 dianas asociadas al MASLD utilizando protocolos de acoplamiento validados. El compuesto potencial se sometió a un análisis de dinámica molecular.

**Resultados:** Se identificaron diecisiete compuestos en el extracto de *C. nutans*. Seis metabolitos, junto con el ácido estearidónico, mostraron propiedades farmacocinéticas favorables. Entre ellos, un derivado del ácido benzoico basado en tiofeno y la tagalsina Q mostraron fuertes afinidades de unión hacia CCR2, ChREBP y SGLT2, con perfiles de interacción comparables a los de los ligandos nativos.

**Conclusiones:** Los metabolitos de *C. nutans* poseen una bioactividad prevista contra CCR2, ChREBP y SGLT2, lo que respalda su potencial utilidad como agentes fitoterapéuticos multiobjetivos para la MASLD.

**Palabras Clave:** *Acanthaceae*; acoplamiento molecular; ADMET predicción; *Clinacanthus nutans*; compuestos naturales; hepatitis.

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## AUTHOR INFO

ORCID:

[0000-0002-3535-2271](https://orcid.org/0000-0002-3535-2271) (NH)

[0000-0001-6461-5782](https://orcid.org/0000-0001-6461-5782) (AM)

[0000-0002-7005-0025](https://orcid.org/0000-0002-7005-0025) (VPK)

[0000-0002-0584-5040](https://orcid.org/0000-0002-0584-5040) (KUH)

[0000-0003-1415-6033](https://orcid.org/0000-0003-1415-6033) (MM)

[0000-0002-5717-8251](https://orcid.org/0000-0002-5717-8251) (HW)

[0000-0002-3722-8979](https://orcid.org/0000-0002-3722-8979) (NU)

**Abbreviations:** 11 $\beta$ -HSD1: 11 $\beta$ -hydroxysteroid dehydrogenase type I; ACC: acetyl-CoA carboxylase; *C. nutans*: *Clinacanthus nutans*; CCL2: chemokine ligand 2; CCR: C-C chemokine receptor; ChREBP: carbohydrate response element-binding protein; FFAR: free fatty acid receptor; FXR: Farnesoid X receptor; GAFF: General Amber Force Field; GLP1: glucagon-like peptide 1; GPR: G protein-coupled receptor; HMGCoA: 3-hydroxy-3-methylglutaryl coenzyme-A; LC-MS/MS: liquid chromatography-mass spectrometry; LXR: liver X receptor; MASH: metabolic dysfunction-associated steatohepatitis; MASLD: metabolic dysfunction-associated steatotic liver disease; MD: molecular dynamics; MM/GBSA: molecular mechanics generalized Born surface area; MM/PBSA: molecular mechanics Poisson-Boltzmann surface area; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; PME: particle mesh Ewald; PPARs: peroxisome proliferator-activated receptors; RMSD: root mean square deviation; SGLT2: sodium-glucose cotransporter-2; SREBP1c: sterol regulatory element-binding protein 1c; T204D: TGF- $\beta$  receptor type 1 kinase domain; TACE: TNF $\alpha$  converting enzyme; TNF $\alpha$ : tumor necrosis factor- $\alpha$ ; UPLC-MS: ultra-high-performance liquid chromatography-mass spectrometry.

## INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) represents a redefined clinical spectrum previously referred to as non-alcoholic fatty liver disease (NAFLD), reflecting its association with cardiometabolic risk factors. It affects approximately 30% of the global adult population (Miao et al., 2024), with progression ranging from simple steatosis to steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. Despite this widespread prevalence, therapeutic options remain insufficient, with only resmetirom approved for MASLD-related steatohepatitis (Tacke et al., 2024).

The multifactorial pathogenesis of MASLD, driven by lipid and glucose dysregulation, inflammation, apoptosis, and fibrosis, complicates the development of effective monotherapy (Portincasa et al., 2024; Tacke et al., 2023). Consequently, research has pivoted toward agents capable of modulating multiple disease-related pathways (Xu et al., 2022). Natural products, particularly polyphenol-rich plant extracts, have demonstrated multi-target actions, making them promising candidates for MASLD treatment (Ore and Akinloye, 2021).

*Clinacanthus nutans* (Acanthaceae), a medicinal plant native to Southeast Asia, has demonstrated potential therapeutic effects, including anti-inflammatory, anti-diabetic, and hepatoprotective properties in preclinical models (Chia et al., 2022; Mustika et al., 2023; Zhang et al., 2023). However, its molecular interactions with specific MASLD targets remain underexplored. This study was conducted to identify the phytochemical constituents of *C. nutans* ethanolic extract and evaluate their drug-likeness, pharmacokinetic properties, and binding affinities against validated MASLD targets using computational methods.

## MATERIAL AND METHODS

### Plant material and extract preparation

Aerial parts of *Clinacanthus nutans* L. were collected from Balai Materia Medica Batu, East Java, Indonesia (-7.867547063098887, 112.51965390340497). The plant identity was confirmed, and voucher specimens were deposited (Batch No. 220525.DGS.F.MMB.002). The

dried material was macerated in 70% ethanol, filtered, and concentrated using a rotary evaporator.

### LC-MS/MS identification

Metabolite profiling was performed using UPLC-MS (Waters ACQUITY H-Class, Xevo G2-S QTOF), operating in positive ESI mode. Solid-phase extraction was applied prior to analysis. Metabolites were identified by comparing MS/MS spectra to PubChem references, with a <5 ppm error threshold (Mutiah and Rachmawati, 2024).

### Drug-likeness filtering and ADMET prediction

SMILES strings of identified compounds were evaluated using SwissADME (<http://www.swissadme.ch/>) (Daina et al., 2017; Jia et al., 2020). Compounds meeting at least one criterion (Lipinski, Ghose, or Veber rules) proceeded to pharmacokinetic profiling, which included gastrointestinal (GI) absorption, blood-brain barrier (BBB) penetration, cytochrome P450 (CYP) inhibition, and P-glycoprotein (P-gp) interaction. Toxicological risks were assessed using ProTox 3.0 (Banerjee et al., 2024; Daina et al., 2017).

### Target selection and protein preparation

Twenty MASLD-relevant targets were selected based on literature evidence (Xu et al., 2022). Crystal structures were obtained from RCSB PDB. Protein preparation included the chain correction, hydrogen addition, cofactor preservation, and electrostatics calculation using UCSF Chimera and Modeller (Pettersen et al., 2004; Webb and Sali, 2016).

### Molecular docking and interaction analysis

Docking validation was performed by reproducing native ligand poses (RMSD  $\leq$  2.0 Å) using SMINA and DockRMSD (Bell and Zhang, 2019; Koes et al., 2013). Ligand-protein interactions were visualized in Chimera and analyzed using PoseEdit. Binding energies and key residue interactions were compared to those of reference ligands, including several agents that have been included in the pharmacological management of MASLD, such as empagliflozin, an SGLT2 inhibitor,

and saroglitazar, an antifibrotic agent (Schöning-Stierand et al., 2022).

### Molecular dynamics simulation

To obtain reliable docking poses for molecular dynamics (MD) simulations, the top five conformers with the lowest docking scores were selected. The MD protocol was adapted from the procedure described by Mei et al. (2021) using the AMBER22 package. Topology and coordinate files for the solvated protein-ligand complexes were generated using the tleap module in the Amber suite. The protein was parameterized with the Amber ff14SB force field, while the ligand was treated with the General Amber Force Field (GAFF). Partial atomic charges for the ligand were assigned using the AM1-BCC method via the antechamber module (Mei et al., 2021).

The complex was solvated in a cubic TIP3P water box with a 10 Å buffer from the solute to the box edges. Counter-ions ( $\text{Cl}^-$  or  $\text{Na}^+$ ) were added to neutralize the system. Energy minimization was performed in three stages using the sander module, with each stage consisting of 5,000 steps of steepest descent followed by 5,000 steps of conjugate gradient minimization. A convergence criterion of  $0.2 \text{ kcal mol}^{-1}$  was applied (Mei et al., 2021).

The system was gradually heated from 0 K to 298 K over 500 ps under the NVT ensemble, followed by a 1 ns equilibration under the NPT ensemble. The production run was performed at 298 K and 1 atm. Covalent bonds involving hydrogen atoms were constrained using the SHAKE algorithm. Long-range electrostatic interactions were calculated using the particle mesh Ewald (PME) method, and short-range van der Waals interactions were treated with a 10 Å cutoff. A 2 fs integration time step was used throughout the simulation. The production phase was carried out for 1 ns, and trajectory snapshots were saved every 1 ps. The binding free energy ( $\Delta G$ ) was calculated using the molecular mechanics generalised Born surface area (MM/GBSA) and molecular mechanics Poisson-

Boltzmann surface area (MM/PBSA) approaches from the production phase trajectory, as implemented in the MMPBSA.py script (Mei et al., 2021).

### Data analysis

All identified compounds of the LC/MS-MS analysis were listed in a table and were subject to drug-likeness screening. Only compounds that passed the screening would be further analyzed for their predicted ADME properties. The prediction of hepatotoxicity, nephrotoxicity, neurotoxicity, and carcinogenicity of these compounds was also analyzed and depicted in a figure.

The target molecules and the ligands were listed on a table. Only those having crystal structures and native ligands that had been retrieved in the RSCB PDB and PubChem proceeded to the docking process. The native ligands are used to identify the binding sites and validate the binding interaction. They also serve as references. The docking scores of the *C. nutans* compounds, predicting their binding affinities to the target proteins, are listed in a table and compared to the docking scores of their respective native ligands. Only compounds whose docking scores were close to those of the native ligand were then continued to binding pose analysis. At this step, the binding poses of the native ligands to the molecular target at specific key amino acid residues were used as a reference for predicting the compounds' activity. Those with similar key interactions to the native ligands were considered potentially active compounds. The potential compound was subsequently subjected to a molecular dynamics simulation.

## RESULTS AND DISCUSSION

### Metabolite profiling

The ion chromatogram obtained from mass spectroscopy is shown in Fig. 1. Seventeen metabolites, including flavonoids, diterpenoids, glycosides, and alkaloids, were identified in *C. nutans* extract (Table 1).

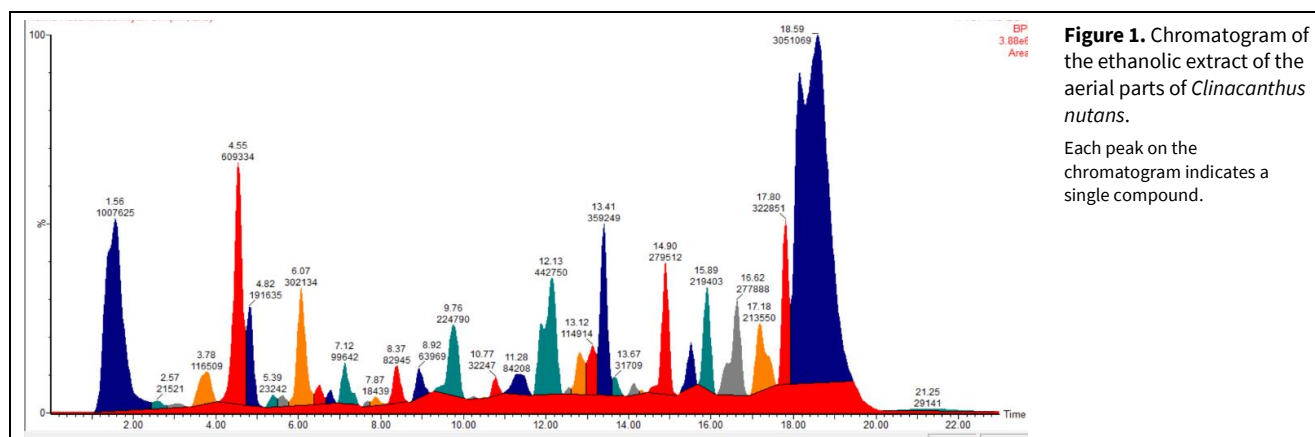


**Figure 1.** Chromatogram of the ethanolic extract of the aerial parts of *Clinacanthus nutans*.

Each peak on the chromatogram indicates a single compound.

**Table 1.** Prediction of compounds in the ethanolic extract of the aerial parts of *Clinacanthus nutans* by liquid chromatography-tandem mass spectrophotometry analysis.

| Retention time (min) | Area (%) | Measured mass (m/z) | Calculated mass (m/z) | Formula                                                        | Compound                                                                                                                                                                                                     | Class                      |
|----------------------|----------|---------------------|-----------------------|----------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|
| 1.56                 | 6.172%   | 380.0800            | 380.0815              | C <sub>21</sub> H <sub>16</sub> O <sub>5</sub> S               | 2-[3-[5-(1-Carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid                                                                                                                                             | Carboxylic acid derivative |
| 3.78                 | 1.415%   | 393.1714            | 393.1713              | C <sub>16</sub> H <sub>27</sub> NO <sub>10</sub>               | 6'-Apiosyllotaustralin                                                                                                                                                                                       | Cyanogenic glycosides      |
| 4.55                 | 5.691%   | 564.1566            | 564.1557              | C <sub>26</sub> H <sub>28</sub> O <sub>14</sub>                | Isoschaftoside                                                                                                                                                                                               | Flavone                    |
| 4.82                 | 1.885%   | 534.1450            | 534.1425              | C <sub>25</sub> H <sub>26</sub> O <sub>13</sub>                | Apigenin 6-C-alpha-L-arabinopyranosyl-8-C-beta-D-xylopyranoside                                                                                                                                              |                            |
| 6.07                 | 3.019%   | 196.1186            | 196.1178              | C <sub>11</sub> H <sub>16</sub> O <sub>3</sub>                 | Isocalendin                                                                                                                                                                                                  | Apocarotenoids (β-)        |
| 7.12                 | 1.046%   | 439.2494            | 439.2496              | C <sub>19</sub> H <sub>37</sub> NO <sub>10</sub>               | (3R,4S,5R,6R)-2-[(2R,3S,4R,5R,6S)-6-[2-(butylamino)propyl]-4,5-dihydroxy-2-(hydroxymethyl)oxan-3-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol                                                                   | Glycoside                  |
| 8.37                 | 1.003%   | 274.2020            | 273.2011              | C <sub>18</sub> H <sub>26</sub> O <sub>2</sub>                 | Tagalsin Q                                                                                                                                                                                                   | Erythroxyane diterpenoids  |
| 8.92                 | 0.710%   | 362.2073            | 362.2073              | C <sub>23</sub> H <sub>26</sub> N <sub>2</sub> O <sub>2</sub>  | Phenaziterpene B                                                                                                                                                                                             | Phenazine alkaloids        |
| 9.76                 | 1.119%   | 290.1967            | 290.1960              | C <sub>18</sub> H <sub>26</sub> O <sub>3</sub>                 | Tuberatolide A                                                                                                                                                                                               | Phytane diterpenoids       |
| 12.13                | 3.404%   | 276.2177            | 276.2168              | C <sub>18</sub> H <sub>28</sub> O <sub>2</sub>                 | Stearidonic acid                                                                                                                                                                                             | Unsaturated fatty acids    |
| 13.41                | 3.309%   | 495.3400            | 495.3445              | C <sub>26</sub> H <sub>45</sub> N <sub>3</sub> O <sub>6</sub>  | N-((2S,3S,5R)-6-(butylamino)-3-hydroxy-1-(isopropylamino)-5-methyl-6-oxohexan-2-yl)-4-methoxy-3-(3-methoxypropoxy)benzamide                                                                                  | Alkaloid                   |
| 14.90                | 3.075%   | 523.3720            | 523.3673              | C <sub>24</sub> H <sub>45</sub> N <sub>9</sub> O <sub>4</sub>  | (2S)-2-acetamido-N-[(2S)-1-[[[(1E,2S)-1-(carbamoylhydrazinylidene)-5-(diaminomethylideneamino)(113C)pentan-2-yl]amino]-3-cyclohexyl-1-oxopropan-2-yl]-4-methylpentanamide                                    | Peptide                    |
| 15.89                | 2.806%   | 610.4669            | 610.4644              | C <sub>39</sub> H <sub>62</sub> O <sub>5</sub>                 | [3-hydroxy-2-[(6Z,9Z,12Z,15Z)-octadeca-6,9,12,15-tetraenoyl]oxypropyl](9Z,12Z,15Z)-octadeca-9,12,15-trienoate                                                                                                | Diacylglycerol             |
| 16.62                | 2.478%   | 610.4669            | 610.4644              | C <sub>40</sub> H <sub>66</sub> S <sub>2</sub>                 | 1-Tetradecyl-4-[(4-tetradecylphenyl)disulfanyl]benzene                                                                                                                                                       | Catechols with side chains |
| 17.18                | 1.950%   | 588.4832            | 588.4832              | C <sub>37</sub> H <sub>66</sub> O <sub>5</sub>                 | Triproxollin                                                                                                                                                                                                 | Acetogenins                |
| 17.80                | 3.558%   | 953.6138            | 953.6127              | C <sub>47</sub> H <sub>83</sub> N <sub>7</sub> O <sub>13</sub> | 18-[2-[2-[2-[2-[2-[[[(5S)-5-carboxy-5-[[3-[3-(1H-imidazol-5-yl)propanoylamino]-3-methyl-2-oxobutyl]amino]pentyl]amino]-2-oxoethoxy]ethoxy]ethylamino]-2-oxoethoxy]ethoxy]ethylamino]-18-oxooctadecanoic acid | Fatty acid                 |
| 18.59                | 24.3125% | 662.4537            | 662.4499              | C <sub>44</sub> H <sub>58</sub> N <sub>2</sub> O <sub>3</sub>  | 4-[5a,5b,8,8,11a-pentamethyl-1-prop-1-en-2-yl-3a-(2-pyridin-2-ylethylcarbamoyl)-1,2,3,4,5,6,7,7a,11,11b,12,13,13a,13b-tetradecahydrocyclopenta[a]chrysen-9-yl]benzoic acid                                   |                            |

Flavones, such as isoschaftoside, and diterpenes, like tagalsin Q and tuberatolide A, were notable for their reported roles in metabolic modulation. Flavones have shown beneficial effects on dysregulated lipid metabolism, oxidative stress, and inflammation in a MASLD model (Li et al., 2020a). UPLC-QTOF/MS

analysis of the ethanolic extract from the aerial parts of *C. nutans* grown in Malaysia identified several flavones, one of which was isoschaftoside, a compound known to induce autophagy, thereby reversing MASLD (Mustapa et al., 2015; Su et al., 2022).

Diterpenoid constituents in *C. nutans* exhibited potential activity against MASLD/MASH, as reported in several experimental studies on diterpenoids, e.g., ginkgolide derived from *Ginkgo biloba* L. (Yao and Liu, 2023). In contrast to our findings, Susanti et al. (2013) did not identify terpenoids in the ethanol extracts of *C. nutans* leaves planted in the same area. The two terpenoids identified in this study, tagalsin Q and tuberatulide A, were likely contained in the stem parts since we investigated the aerial parts of *C. nutans*.

### Drug-likeness and ADMET prediction

Six compounds passed the screening criteria, including 1) (2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid, 2) isocalendin, 3) tagalsin Q, 4) phenaziterpene B, 5) tuberatulide A, and 6) N-((2S,3S,5R)-6-(butylamino)-3-hydroxy-1-(isopropylamino)-5-methyl-6-oxohexan-2-yl)-4-methoxy-3-(3-methoxypropoxy) benzamide. Drug-likeness and ADME profiles are presented in Table S1. All exhibited high GI absorption. All compounds except compounds 1 and 6 demonstrated BBB permeability. Compound 6 was the only candidate identified as a P-gp substrate, which may impede its intracellular accumulation and complicate co-administration with P-gp inhibitors. Given the established role of P-gp and other ATP-binding cassette (ABC) transporters in pharmacoresistance, further computational and experimental investigations are warranted to evaluate compound interactions with various efflux transporters.

Several compounds also showed potential to inhibit key cytochrome P450 enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6), indicating a risk of herb-drug interactions that could elevate plasma drug levels and compromise safety. Besides common drugs like ibuprofen and warfarin, which are CYP2C6 substrates, and codeine, a CYP2D6 substrate, caffeine, primarily metabolized by CYP1A2, may also be affected by *C. nutans* metabolites (Cheng et al., 2023). The predictive molecular structures and toxicity profiles of these compounds are depicted in Fig. 2. Compounds 1 and 3 were flagged for hepatotoxicity, while compounds 1 and 6 may pose a risk of nephrotoxicity. Future studies should include in vitro hepatocyte or renal cell assays to verify these findings. Neurotoxicity is likely to occur upon exposure to compound 3, given its BBB-permeant profile. Only compound 2 was shown to possess carcinogenicity properties, although the predictive model indicated a probability of 0.52.

None of the six compounds is from the flavonoid class. Previous *in silico* studies of *C. nutans* constituents have predominantly analyzed compounds of this class, focusing on their potential inhibitory activities toward biological enzymes involved in chronic inflammation (Narayanaswamy et al., 2016). Another study reported

their potential interaction with aldose reductase, glucokinase, and GSK3 $\beta$  proteins, suggesting antidiabetic activity (Putri et al., 2023). Of many flavonoids, only a few compounds exhibited drug-like properties according to Lipinski's rules. These include clinacoside A, B, C, cycloclinacoside, and apigenin. (Narayanaswamy et al., 2016; Putri et al., 2023). In this study, Isoschaftoside, an isomer of schaftoside and a flavone C-glycoside of apigenin, exhibited violation of all rules. Schaftoside was also reported to violate drug-likeness rules (Narayanaswamy et al., 2016). Nevertheless, intraperitoneal injection of isoschaftoside was shown to improve histological and biochemical parameters of MASLD in high-fat diet-induced mice (Su et al., 2022).

### Validation of target proteins for molecular docking

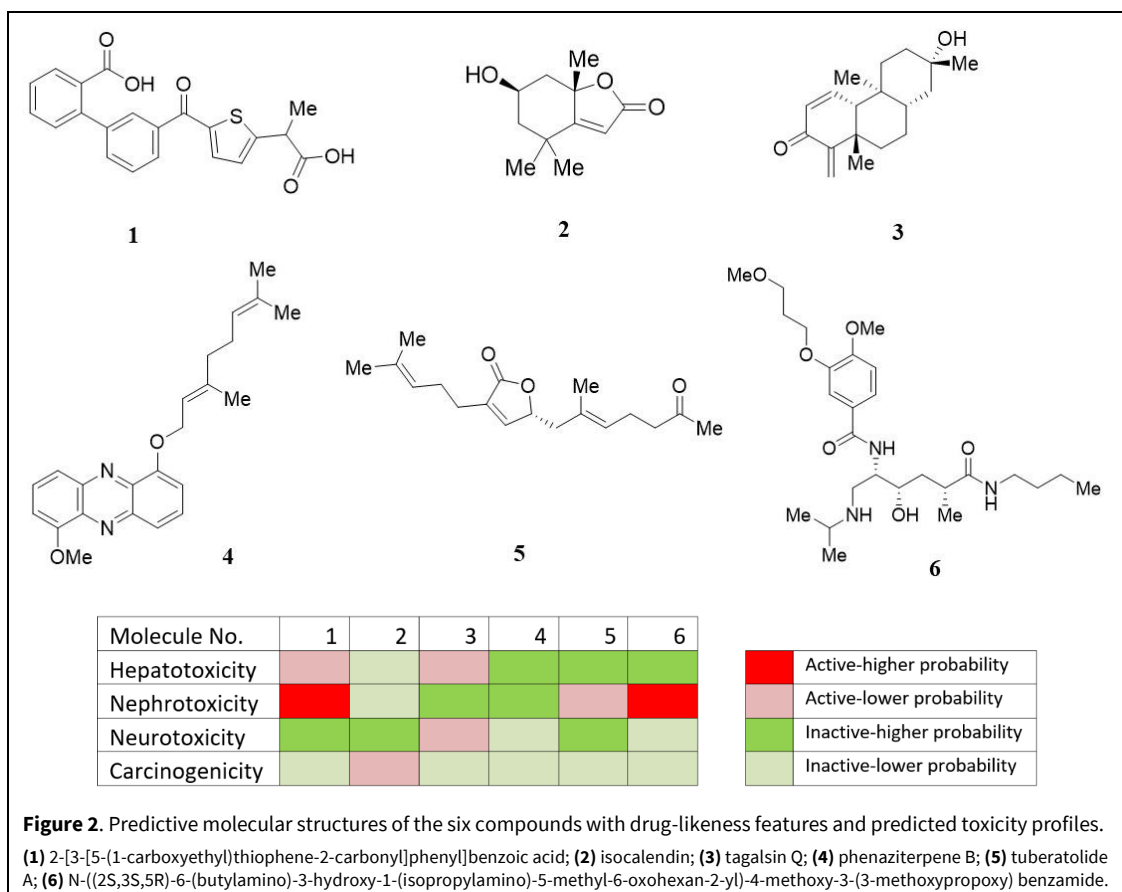
Twenty target proteins were validated, including nuclear receptors, membrane receptors, transcription factors, signal transduction modulators, enzymes, and a transporter. The RMSD values ranged from 0.234–1.848 Å (Table 2). Most ligands bound to targets directly involved in cellular and molecular pathways of liver pathologies have entered clinical investigation stages; however, only a liver-selective agonist for thyroid receptor- $\beta$  has been approved for treating fibrosis in non-cirrhotic MASH (Tacke et al., 2024; Xu et al., 2022). Several targets have been implicated in lipid and carbohydrate metabolism, including 3-hydroxy-3-methylglutaryl coenzyme-A reductase, glucagon-like peptide 1, and sodium-glucose cotransporter-2 (SGLT2). Their ligands were used for dyslipidemia and diabetes and were included in the MASLD guidelines (Tacke et al., 2024).

Peroxisome proliferator-activated receptors (PPARs)  $\alpha/\delta/\gamma$  agonists have shown favorable efficacy in histological improvement, despite persistent issues with adverse effects (Tacke et al., 2024). Numerous pathologically affected pathways in MASH are regulated by PPARs, including those involved in hepatic lipid and glucose metabolism and the immune-inflammatory response. Their activation in multiple organs modulates the crosstalk between the adipose tissue, intestine, and the liver. Moreover, the activation of PPARs in the heart, pancreas, and skeletal muscle is expected to nullify cardiometabolic factors in MASH (Staels et al., 2023). Farnesoid X receptor (FXR) is a promising target. According to the early findings of a phase III clinical trial, obeticholic acid, a synthetic ligand of FXR, showed an antifibrotic effect in patients with pre-cirrhotic NASH (Sanyal et al., 2023). The liver X receptor (LXR) inhibitor oltipraz was investigated in a phase II study and showed antisteatotic activity. One of the functions of LXR is to transactivate SREBP1c, thereby inducing the expression of lipogenic genes (Xu et al., 2022).

**Table 2.** PDB ID, targets, resolution, and RMSD values of the reproduced binding pose on 20 targets.

| No.                                                          | PDB ID | Target and native ligand                                                                                                                                                  | Resolution (Å) | RMSD (Å)                                  | Ref.                           |
|--------------------------------------------------------------|--------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|-------------------------------------------|--------------------------------|
| <b>Nuclear receptor</b>                                      |        |                                                                                                                                                                           |                |                                           |                                |
| 1                                                            | 6LXB   | X-ray structure of human <b>PPARα</b> ligand binding domain- <b>saroglitazar</b> co-crystals obtained by soaking                                                          | 2.36           | 0.342                                     | (Kamata et al., 2020)          |
| 2                                                            | 8HUL   | X-ray structure of human <b>PPARδ</b> ligand binding domain- <b>lanifibranor</b> co-crystals obtained by co-crystallization                                               | 2.46           | 0.544                                     | (Kamata et al., 2023)          |
| 3                                                            | 8HUP   | X-ray structure of human <b>PPARγ</b> ligand binding domain- <b>seladelpar</b> -SRC1 coactivator peptide co-crystals obtained by co-crystallization                       | 2.36           | 1.735                                     | (Kamata et al., 2023)          |
| 4                                                            | 6ITM   | Crystal structure of <b>FXR</b> in complex with agonist <b>XJ034</b>                                                                                                      | 2.5            | 0.497                                     | (Xia et al., 2020)             |
| 5                                                            | 3L0E   | X-ray crystal structure of a potent <b>LXR</b> modulator in complex with <b>GSK2033</b>                                                                                   | 2.3            | 0.474                                     | (Zuercher et al., 2010)        |
| 6                                                            | 1N46   | Crystal structure of human <b>TRβ</b> ligand-binding domain complexed with a potent subtype-selective thyromimetic                                                        | 2.2            | 0.346                                     | (Dow et al., 2003)             |
| <b>Membrane receptor</b>                                     |        |                                                                                                                                                                           |                |                                           |                                |
| 7                                                            | 6X1A   | Non peptide agonist <b>PF-06882961</b> , bound to <b>GLP-1 receptor</b>                                                                                                   | 2.5            | 1.769                                     | (Zhang et al., 2020)           |
| 8                                                            | 4MBS   | Crystal structure of the <b>CCR5</b> chemokine receptor in complex with <b>maraviroc</b>                                                                                  | 2.71           | 0.838                                     | (Tan et al., 2013)             |
| 9                                                            | 5T1A   | Structure of <b>CCR2</b> with orthosteric antagonist (in complex with <b>BMS-681</b> ) and allosteric antagonist (in complex with <b>CCR2-RA-[R]</b> )                    | 2.81           | 1.137 (orthosteric)<br>0.333 (allosteric) | (Zheng et al., 2016)           |
| 10                                                           | 8IYS   | <b>TUG891</b> -bound <b>FFAR4</b> in complex with Gq                                                                                                                      | 2.95           | 1.284                                     | (Yin et al., 2023)             |
| 11                                                           | 5KW2   | The extra-helical binding site of <b>GPR40</b> and the structural basis for allosteric agonism and incretin stimulation                                                   | 2.76           | 0.716                                     | (Ho et al., 2018)              |
| <b>Transcription factor and signalling pathway modulator</b> |        |                                                                                                                                                                           |                |                                           |                                |
| 12                                                           | 7JRA   | Human <b>TNF-α</b> in complex with <b>2-[5-(3-chloro-4-[[[(1R)-1-(2-fluorophenyl)ethyl]amino]quinolin-6-yl]pyrimidin-2-yl]propan-2-ol</b>                                 | 2.1            | 0.234                                     | (Xiao et al., 2020)            |
| 13                                                           | 8BTQ   | Small molecule stabilizer for <b>14-3-3/ChREBP</b> (Cmd1-soaking)                                                                                                         | 1.6            | 1.284                                     | (Katz et al., 2025)            |
| <b>Enzyme</b>                                                |        |                                                                                                                                                                           |                |                                           |                                |
| 14                                                           | 5KKN   | Crystal structure of human <b>ACC2</b> BC domain in complex with <b>ND-646</b> , the primary amide of ND-630                                                              | 2.6            | 0.97                                      | (Harriman et al., 2016)        |
| 15                                                           | 8EYK   | Atomic model of the core modifying region of human <b>fatty acid synthase</b> in complex with <b>TVB-2640</b>                                                             | 2.7            | 0.486                                     | (Hasan et al., 2023)           |
| 16                                                           | 4C7J   | <b>11b-Hydroxysteroid dehydrogenase type I</b> in complex with <b>inhibitor</b>                                                                                           | 2.16           | 0.86                                      | (Goldberg et al., 2014)        |
| 17                                                           | 1HWK   | Complex of the catalytic portion of <b>human HMG-CoA reductase</b> with <b>atorvastatin</b>                                                                               | 2.22           | 0.675                                     | (Istvan and Deisenhofer, 2001) |
| 18                                                           | 3LEA   | Crystal structure of the catalytic domain of <b>TACE</b> with <b>isoindolinone-biphenyl-hydantoin</b> inhibitor                                                           | 2.00           | 1.848                                     | (Yu et al., 2010)              |
| 19                                                           | 6B8Y   | <b>TGF-β receptor type 1 kinase</b> domain (T204D) in complex with <b>N-(3-fluoropyridin-4-yl)-2-[6-(trifluoromethyl)pyridin-2-yl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine</b> | 1.65           | 0.383                                     | (Harikrishnan et al., 2018)    |
| <b>Transporter</b>                                           |        |                                                                                                                                                                           |                |                                           |                                |
| 20                                                           | 7VSI   | Structure of human <b>SGLT2</b> -MAP17 complex bound with <b>empagliflozin</b>                                                                                            | 2.95           | 1.103                                     | (Niu et al., 2022)             |

ACC: acetyl-CoA carboxylase; CCR: C-C chemokine receptor; ChREBP: carbohydrate response element-binding protein; FFAR: free fatty acid receptor; FXR: farnesoid X receptor; GLP-1: glucagon-like peptide-1; GPR40: G protein coupled receptor 40; HMG-CoA: hydroxymethylglutaryl-coenzyme-A; LXR, liver X receptor; PDB: protein data bank; PPAR: peroxisome proliferator-activated receptor; Ref.: reference; RMSD: root mean square deviation; SGLT2: sodium-glucose cotransporter-2; TACE: tumor necrosis factor α-converting enzyme; TNF-α: tumor necrosis factor α; TRβ: thyroid receptor-β.



*De novo* lipogenesis is a promising therapeutic target. Orlistat, a fatty acid synthase inhibitor, has passed phase III trials (Hasan et al., 2024), whereas acetyl-CoA carboxylase (ACC) inhibitors have not entered phase III trials (Hasanatuludhhiyah et al., 2025). 11 $\beta$ -Hydroxysteroid dehydrogenase type I (11 $\beta$ -HSD1), an enzyme that serves as a cortisone-to-cortisol converter, amplifies the impact of cortisol on carbohydrate and lipid metabolism in the liver, leading to systemic and hepatic insulin resistance and promoting hepatic steatosis and subsequent pathological processes. The findings of an early-phase clinical study suggest a favorable safety profile for the 11 $\beta$ -HSD1 inhibitor, whereas its efficacy awaits validation in a currently ongoing Phase II trial (Kim et al., 2023).

The free fatty acid receptor (FFAR)1/G protein-coupled receptor (GPR) 40 and FFAR4/GPR 120 mediate the action of omega-3 in reducing steatosis and inflammation in patients with MASLD (Secor et al., 2021). Several targets primarily act in inflammatory pathways. Upon binding to chemokine ligand-2 (CCL2), the C-C chemokine receptor (CCR) 2 mediates the migration of circulating monocytes into the liver, whereas CCR5 mediates the migration and proliferation of lymphocytes and hepatic stellate cells, thereby sustaining the inflammatory milieu and promoting fibrosis. However, the dual CCR2/5 antagonist cenicriviroc failed to show any substantial

improvement in fibrosis in a phase III clinical trial (Lefere et al., 2020). Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is one of the inflammatory cytokines highly involved in MASH pathogenesis, regulating inflammation, cell death, and fibrosis (Lu et al., 2022).

Several targets are still under investigation in the preclinical stage. The carbohydrate-responsive element-binding protein (ChREBP) is a transcription factor that activates lipogenic genes in response to carbohydrates (Katz et al., 2025). TNF $\alpha$ -converting enzyme, also known as disintegrin and metalloproteinase 17, plays a crucial role in regulating the availability and activity of TNF- $\alpha$ . Its inhibitor, marimastat, prevented hepatocarcinogenesis in mice with steatohepatitis induced by a Western diet and carbon tetrachloride (Srinivas et al., 2024).

## Docking results

The docking scores for the six compounds and stearidonic acid are listed in Table 3. Stearidonic acid was also included in docking analysis since its drugability has been established in previous studies (Guil-Guerrero et al., 2007).

Molecular docking identified strong affinities for CCR2, ChREBP, and SGLT2. Compound 1 (2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid) and tagalsin Q showed docking scores close to or

surpassing those of native ligands. These compounds established hydrogen bonding and van der Waals interactions with key residues implicated in ligand stabilization, as indicated by interaction poses (Fig. 3).

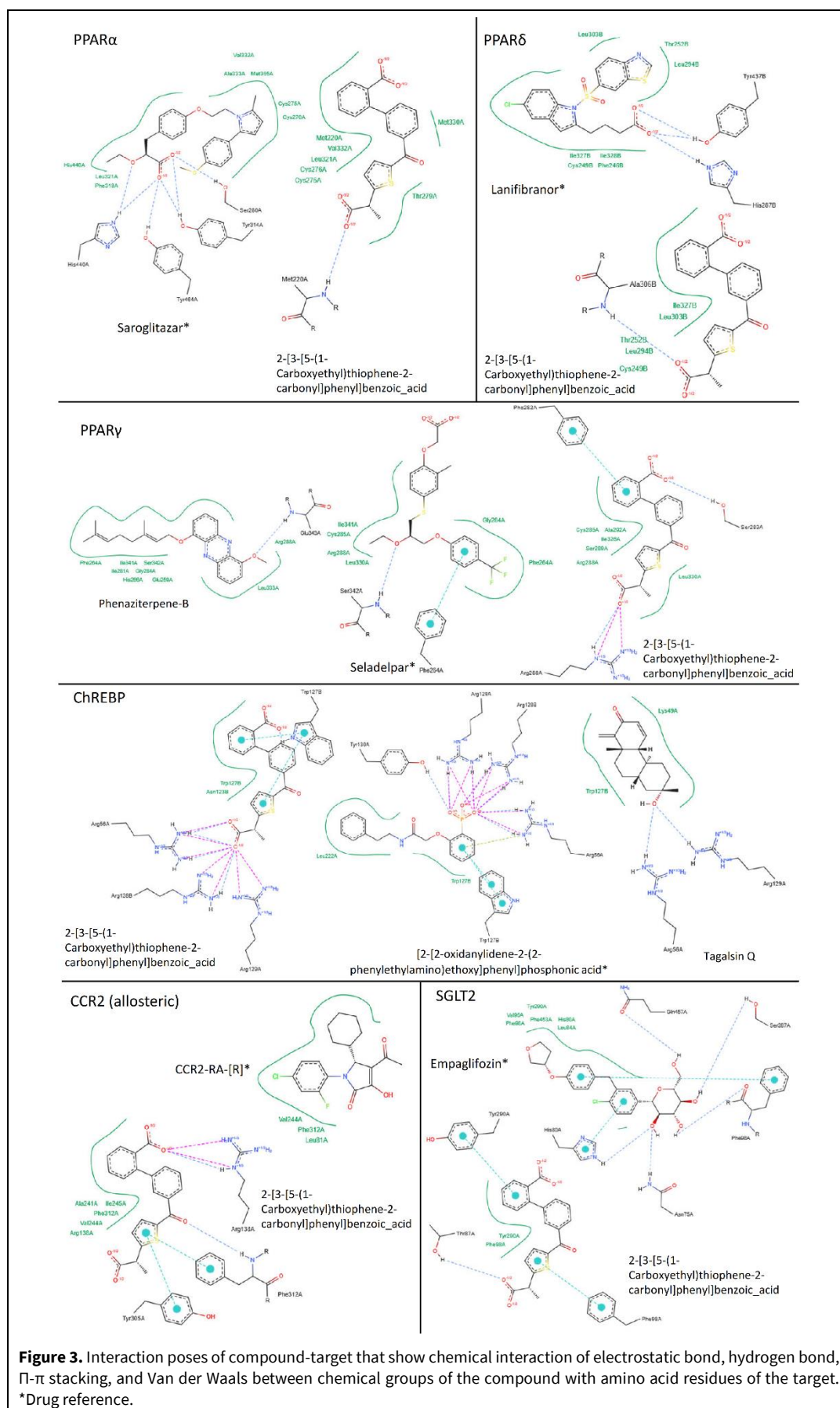
2-[3-[5-(1-Carboxyethyl)thiophene-2-carbonyl]phenyl] benzoic acid had higher docking scores for PPAR $\alpha$ , - $\delta$ , and - $\gamma$ , LXR $\alpha$ , and ChREBP than those of native ligands. Furthermore, its docking score was close to that of the native ligand for CCR2 (allosteric)

and SGLT2, indicating good predictive activity for these targets. Phenaziterpene B was also predicted to have good activity against PPAR $\gamma$ . Tagalsin Q had a higher docking score for ChREBP and was close to the docking score of the native ligand for 11 $\beta$ -HSD1. None of the compounds had docking scores close to those of native ligands for other targets. The interaction poses are depicted in Fig. 3. The chemical interactions of the compounds with the amino acid residues of the targets are presented in Table S2.

**Table 3.** Docking scores of *Clinacanthus nutans* metabolites on metabolic dysfunction-associated steatotic liver-targeted proteins.

| Targeted protein                                     | *Compounds |          |          |          |          |          |          | Native ligand/<br>references |
|------------------------------------------------------|------------|----------|----------|----------|----------|----------|----------|------------------------------|
|                                                      | 1          | 2        | 3        | 4        | 5        | 6        | 7        |                              |
| Nuclear receptors                                    |            |          |          |          |          |          |          |                              |
| PPAR $\alpha$                                        | -9.4067    | -6.57502 | -6.49046 | -8.82988 | -7.51382 | -7.14606 | -6.53121 | 9.1596                       |
| PPAR $\delta$                                        | -9.92129   | -6.93379 | -6.05392 | -9.45301 | -8.33985 | -8.13658 | -6.99004 | -9.90518                     |
| PPAR $\gamma$                                        | -9.24134   | -6.12736 | -6.97615 | -9.56374 | -8.17556 | -7.93798 | -7.55146 | -8.99944                     |
| FXR                                                  | -9.5114    | -7.50079 | -7.71129 | -9.80491 | -8.15623 | -7.77777 | -7.25079 | 12.36829                     |
| LXR                                                  |            |          |          |          |          |          |          |                              |
| Modulator                                            | -10.8947   | -7.87374 | -10.3556 | -10.4461 | -8.36152 | -8.76857 | -7.44823 | -12.94758                    |
| TR $\beta$                                           | -8.99236   | -7.71352 | -6.68283 | -6.2806  | -8.57174 | -2.96424 | -8.63265 | -12.88059                    |
| Membrane receptors                                   |            |          |          |          |          |          |          |                              |
| GLP-1 receptor                                       | -11.2177   | -7.74647 | -8.71304 | -10.432  | -9.28624 | -8.40114 | -7.9447  | -13.18713                    |
| CCR5                                                 | -8.98547   | -6.63029 | -8.4603  | -9.38949 | -7.88871 | -8.04069 | -7.524   | -12.52477                    |
| CCR2 (orthosteric)                                   | -9.2222    | -6.22014 | -8.4333  | -8.59042 | -7.65833 | -7.22182 | -6.17877 | -10.5217                     |
| CCR2 (allosteric)                                    | -10.065    | -7.19819 | -9.05209 | -9.74191 | -8.27691 | -8.04258 | -7.50453 | -11.04719                    |
| FFAR4                                                | -10.1188   | -7.61733 | -9.13149 | -10.0401 | -9.31285 | -6.52457 | -7.90355 | -11.74323                    |
| GPR40                                                | -7.95497   | -6.69374 | -7.81331 | -8.05418 | -7.06835 | -6.45724 | -5.56025 | -9.94263                     |
| Transcription factor and signaling pathway modulator |            |          |          |          |          |          |          |                              |
| TNF $\alpha$                                         | -9.84398   | -8.01341 | -8.05423 | -9.14043 | -9.00013 | -8.18245 | -8.40095 | -13.48021                    |
| ChREBP                                               | -8.07249   | -6.49125 | -7.8297  | -6.78194 | -6.76047 | -6.63431 | -5.70227 | -7.7781                      |
| Enzyme                                               |            |          |          |          |          |          |          |                              |
| ACC2                                                 | -9.84587   | -6.94907 | -8.83101 | -9.13809 | -7.90047 | -7.75035 | -8.07277 | -11.24723                    |
| FASN                                                 | -8.04697   | -4.64914 | -6.42381 | -9.00399 | -7.95396 | -7.07966 | -7.06537 | -11.01704                    |
| 11 $\beta$ -HSD1                                     | -9.12132   | -7.55652 | -9.60184 | -9.35332 | -8.90313 | -7.91138 | -7.28742 | -9.91774                     |
| HMGR                                                 | -8.22377   | -6.52504 | -7.45415 | -7.45143 | -6.18471 | -6.79832 | -5.22733 | -9.96394                     |
| TACE                                                 | -7.3905    | -5.67354 | -6.23123 | -7.80582 | -7.21635 | -6.56509 | -5.66322 | -9.33704                     |
| T204D                                                | -9.88054   | -6.80388 | -8.54968 | -9.33792 | -7.89276 | -7.79391 | -7.10819 | -11.7124                     |
| Transporter                                          |            |          |          |          |          |          |          |                              |
| SGLT2                                                | -11.3459   | -7.88925 | -9.22315 | -10.6422 | -8.67361 | -8.20929 | -7.62138 | -11.86976                    |

*\*(1)* 2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid; *(2)* isocalendin; *(3)* tagalsin Q; *(4)* phenaziterpene B; *(5)* tuberatolide A; *(6)* N-((2S,3S,5R)-6-(butylamino)-3-hydroxy-1-(isopropylamino)-5-methyl-6-oxohexan-2-yl)-4-methoxy-3-(3-methoxypropoxy)benzamide; *(7)* Stearidonic acid. 11 $\beta$ -HSD1: 11b-hydroxysteroid dehydrogenase type I; ACC: acetyl-CoA carboxylase; CCR: C-C chemokine receptor; ChREBP: carbohydrate response element-binding protein; FASN: fatty acid synthase; FFAR: free fatty acid receptor; FXR: farnesoid X receptor; GLP-1: glucagon-like peptide-1; GPR40: G protein-coupled receptor 40; HMGR: HMG CoA reductase; LXR: liver X receptor; PPAR: peroxisome proliferator-activated receptor; SGLT2: sodium-glucose cotransporter-2; T204D: TGF- $\beta$  receptor type 1 kinase domain; TACE: tumor necrosis factor  $\alpha$ -converting enzyme; TNF $\alpha$ : tumor necrosis factor alpha; TR $\beta$ : thyroid hormone receptor-beta.



Significant interactions between PPAR $\alpha$ ,  $\delta$ , and  $\gamma$  and their respective agonists (saroglitazar, lanifibranor, seladelpar) are through hydrogen bonds with several amino acid residues. The carboxyl group of saroglitazar forms hydrogen bonds with Ser 280, Tyr 314, His 440, and His 464 residues of PPAR $\alpha$ . Meanwhile, the carboxyl group of 2-[3-[5-(1-carboxyethyl) thiophene-2-carbonyl]phenyl]benzoic acid interacts with PPAR $\alpha$  through a hydrogen bond with a different amino acid residue, namely Met 220. Similarly, this compound is expected to favorably interact with PPAR $\delta$  and  $\gamma$ , but its activities on these receptors are possibly different from those of well-known agonists, lanifibranor and seladelpar.

The carboxyl groups of 2-[3-[5-(1-carboxyethyl) thiophene-2-carbonyl]phenyl] benzoic acid are predicted to form hydrogen bonds with Arg 288 and Ser 289 residues of PPAR $\gamma$ . It also forms electrostatic bonds with the protonated amines of Arg 288, and a  $\Pi$ - $\pi$  stacking with Phe 282. Phenaziterpene B also shows significant interaction with PPAR $\gamma$  through a hydrogen bond to Glu 343, indicating a potential activity on this receptor. However, it does not resemble seladelpar, which forms a hydrogen bond and a  $\Pi$ - $\pi$  stacking with Ser 342 and Phe 264 of PPAR $\gamma$ , respectively.

C-C chemokine receptor type 2 interacts with an allosteric inhibitor via van der Waals force at Leu 81, Phe 312, and Val 244 residues. The compound 2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid interacted with key residues of CCR2, including Phe312 and Val244, indicating potential allosteric inhibition. These interactions mirror those of known CCR2 antagonists, suggesting plausible anti-inflammatory effects. This compound also forms other types of chemical interactions, including electrostatic and hydrogen bonds, and  $\Pi$ - $\pi$  stacking.

ChREBP is bound to a small molecule stabilizer (compound 1) via electrostatic and hydrogen bonds, and  $\Pi$ - $\pi$  stacking. Compound 1 acts as a molecular glue that stabilizes the ChREBP/14-3-3 protein-protein interaction, preventing the nuclear translocation of this transcription factor and thus averting its transcriptional activity in inducing glucolipotoxicity (Katz et al., 2025). 2-[3-[5-(1-Carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid seems to have parallel activity with compound 1 since it also forms chemical interactions with ChREBP through these types of bonds upon similar amino acid residues.

Tagalsin Q is also predicted to have favorable activity towards ChREBP. In addition to having a higher docking score than compound 1, interaction pose analysis reveals a hydrogen bond between this compound and the Arg56 residue of ChREBP. The chemical interaction of SGLT2-empagliflozin is via hydrogen bonds

and  $\Pi$ - $\pi$  stacking. While 2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid also forms these types of chemical interactions. It similarly interacts with Phe 98, forming a  $\Pi$ - $\pi$  stacking. Therefore, it is expected to have an inhibitory action on SGLT2, like empagliflozin (Niu et al., 2022).

The *in silico* analysis predicts that two metabolites of *C. nutans*, 2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid and tagalsin Q, have pharmacological activities against CCR2, ChREBP, and SGLT2. Notwithstanding the potential pharmacological activities of these compounds in MASLD, findings on probable hepatotoxicity and nephrotoxicity of 2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid and probable hepatotoxicity and neurotoxicity of tagalsin Q should navigate future *in vivo* studies for dose optimization and ascertainment of safety, particularly on these organs. Inhibitory activities of these compounds toward certain subtypes of CYP should raise a hypothetical notion that either *C. nutans* extract or its isolated compounds may have drug interaction issues.

The expressions of CCR2 and CCL2 are elevated in MASLD. CCR2-CCl2 binding is accountable for inflammatory cell infiltration, which leads to the progression into steatohepatitis. CCR2 has been implicated in liver fibrosis through M1 polarization and the activation of hepatic stellate cells (Li et al., 2020b). The CCR2 inhibitor has been documented to attenuate insulin resistance and prevent MASH development (Mulder et al., 2017). Therefore, the predictive inhibitory action of 2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid towards CCR2 is likely to decelerate MASLD progression through the suppression of inflammatory and fibrogenesis pathways. *C. nutans* extract has been shown to reduce inflammatory cytokines in various pathological models with inflammatory backgrounds (Azemi et al., 2021; Ong et al., 2022). This pharmacological activity likely corresponds to the suppression of the CCR2-CCl2 axis and M2 polarization, although experimental evidence is necessary to confirm this. The potential activity of 2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl] benzoic acid and tagalsin-Q in stabilizing the ChREBP/14-3-3 complex and thus retaining this transcriptional factor in the cytosol raises the question of whether these compounds indeed avert the upregulation of lipogenic gene expression in MASLD related to insulin resistance. The findings of this study suggest an alternative mode of action for *C. nutans* as an euglycemic agent, mediated by its inhibitory action on SGLT2. *C. nutans* ethanolic extract has been demonstrated to lower blood glucose in the type-1 DM model by reducing apoptosis of pancreatic  $\beta$ -cells (Mustika et al., 2023).

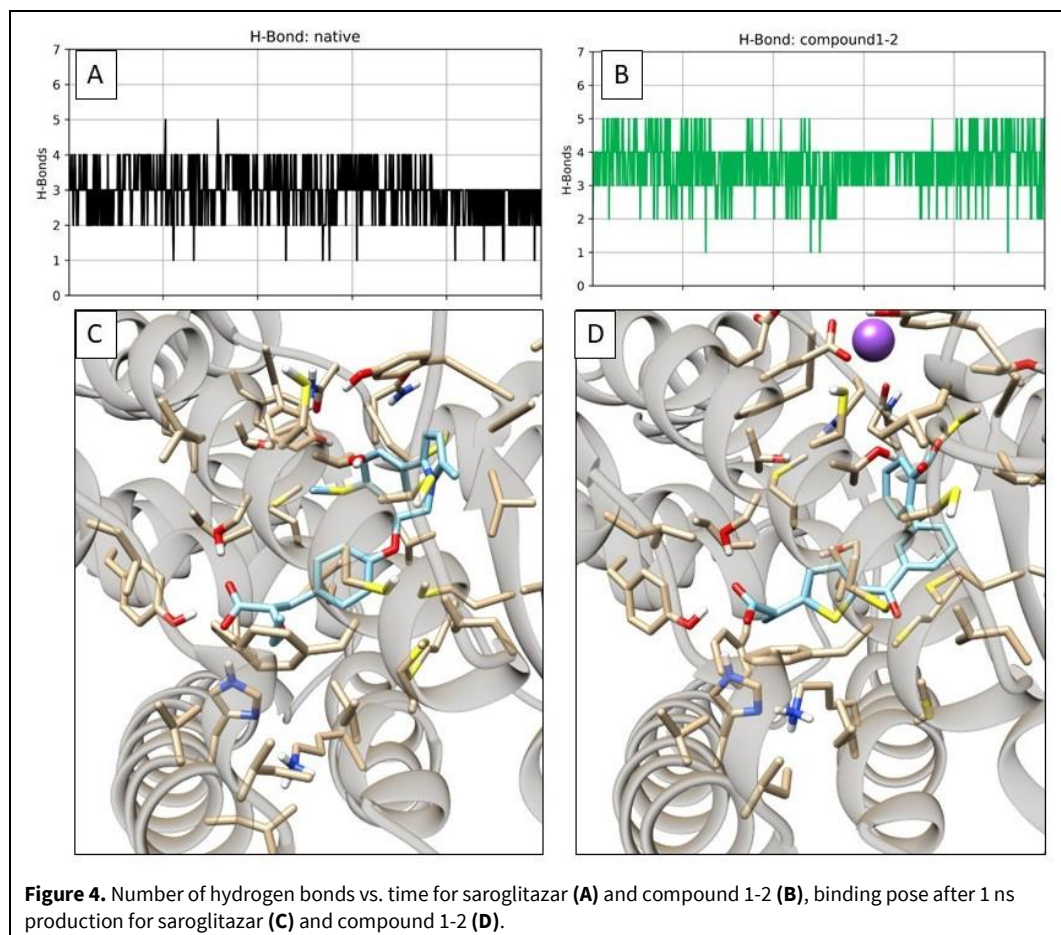


**Figure 4.** Number of hydrogen bonds vs. time for saroglitazar (A) and compound 1-2 (B), binding pose after 1 ns production for saroglitazar (C) and compound 1-2 (D).

SGLT2 inhibitors induce glucosuria, which then alleviates hyperglycemia and insulin resistance. It has been shown to reduce steatosis, liver enzymes, and even fibrosis (Portincasa et al., 2024). Therefore, it is recommended for MASLD with type-2 DM (Tacke et al., 2024).

### Molecular dynamics analysis

Based on the docking scores, 2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid (compound 1) exhibits potential bioactivity as a PPAR $\alpha$  agonist.

However, given the well-known limitations of docking scores in accurately predicting binding affinity, a more rigorous evaluation using molecular dynamics (MD) simulations was performed. Binding free energies were calculated using the MM/GBSA and MM/PBSA methods, as presented in Table S3.

Among the five conformers analyzed, conformer 2 of Compound 1 (Compounds 1-2) consistently displayed the lowest  $\Delta G$  values, indicating it is the most stable among the generated poses. Nevertheless, its binding affinity remains weaker than that of the native ligand, saroglitazar, suggesting limited binding strength. This lower affinity can be attributed to

suboptimal protein–ligand interactions. Although Compound 1 forms a greater number of hydrogen bonds (approximately 3–5) compared to saroglitazar (2–4), the overall interaction complementarity is inferior. Specifically, the hydrophobic region of saroglitazar fits well into the hydrophobic pocket of the binding site, thereby stabilizing the interaction. In contrast, Compound 1 contains two carboxylate groups, one of which occupies the hydrophobic pocket, leading to unfavorable desolvation and electrostatic repulsion. This mismatch likely contributes to the reduced binding affinity observed (Fig. 4).

We successfully screened seven metabolites identified in the ethanolic extract of the aerial parts of *C. nutans*, which also met drug-likeness criteria, for potential activities against validated molecular targets of MASLD. However, while docking scores and RMSD values offer insights into binding affinity and pose stability, they do not directly reflect functional activity. Docking algorithms and scoring functions are based on simplified assumptions that may not fully capture the complexity of biological systems, and an over-reliance on RMSD can be misleading, particularly in light of conformational flexibility (Paggi et al., 2024). Similarly, while molecular dynamics simulations provide additional information on the stability and behavior of

ligand–receptor interactions over time, they remain limited by the accuracy of force fields and the inability to fully model cellular environments (Grewal et al., 2025).

The metabolomic and *in silico* findings support the potential of *C. nutans* metabolites as therapeutic agents for MASLD. The ability of selected compounds to interact with CCR2, ChREBP, and SGLT2 highlights their potential to modulate inflammation, lipogenesis, and glucose uptake—three critical axes in MASLD pathophysiology. Diterpenoids, such as tagalsin Q, may contribute to anti-fibrotic or metabolic regulatory effects, aligning with the known activities of diterpenoid scaffolds in other models. The absence of *in vitro* or *in vivo* validation is a limitation; however, the molecular docking results offer a rational foundation for future experimental designs. Importantly, incorporating reference inhibitors in the docking workflow (e.g., empagliflozin for SGLT2) is recommended to improve benchmarking and translational potential. In follow-up studies, CCR2 target engagement can be validated using fluorescent ligand-displacement binding assays in CCR2-expressing cells. Likewise, ChREBP transcriptional activity can be quantified with a ChoRE-driven luciferase reporter and corroborated by RT-qPCR of canonical target genes.

## CONCLUSION

Selected metabolites from *C. nutans* exhibit favorable pharmacokinetic properties and demonstrate strong predicted interactions with MASLD-associated targets, particularly CCR2, ChREBP, and SGLT2. These findings suggest the potential application of *C. nutans*-derived compounds as multi-target agents in the treatment of MASLD, warranting further pharmacological validation.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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**AUTHOR CONTRIBUTION:**

| Contribution                       | Hasanatuludhhiyah N | Mustika A | Kalanjati VP | Haq KU | Miftahussurur M | Wakuda H | Uemura N |
|------------------------------------|---------------------|-----------|--------------|--------|-----------------|----------|----------|
| Concepts or ideas                  | x                   | x         | x            |        | x               | x        | x        |
| Design                             | x                   | x         | x            | x      | x               | x        | x        |
| Definition of intellectual content | x                   | x         | x            | x      | x               | x        | x        |
| Literature search                  | x                   | x         | x            | x      |                 |          | x        |
| Experimental studies               |                     |           |              |        |                 |          |          |
| Data acquisition                   | x                   |           |              | x      |                 |          |          |
| Data analysis                      | x                   | x         |              | x      |                 |          |          |
| Statistical analysis               |                     |           |              |        |                 |          |          |
| Manuscript preparation             | x                   |           |              |        |                 |          |          |
| Manuscript editing                 | x                   | x         | x            | x      | x               | x        | x        |
| Manuscript review                  | x                   | x         | x            | x      | x               | x        | x        |

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Supplementary data

Table S1. Predicted drug-likeness and ADME.

| Compound | No. of violations |       |       | GI absorption | BBB permeant | Pgp substrate | Inhibitory action |         |        |        |        |
|----------|-------------------|-------|-------|---------------|--------------|---------------|-------------------|---------|--------|--------|--------|
|          | Lipinski          | Ghose | Veber |               |              |               | CYP1A2            | CYP2C19 | CYP2C9 | CYP2D6 | CYP3A4 |
| 1        | 0                 | 0     | 0     | High          | No           | No            | No                | No      | Yes    | No     | No     |
| 2        | 0                 | 0     | 0     | High          | Yes          | No            | No                | No      | No     | No     | No     |
| 3        | 0                 | 0     | 0     | High          | Yes          | No            | No                | Yes     | Yes    | No     | No     |
| 4        | 0                 | 1     | 0     | High          | Yes          | No            | No                | Yes     | Yes    | Yes    | No     |
| 5        | 0                 | 0     | 0     | High          | Yes          | No            | Yes               | Yes     | Yes    | Yes    | No     |
| 6        | 0                 | 3     | 1     | High          | No           | Yes           | No                | No      | No     | No     | No     |

(1) 2-[3-[5-(1-carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid; (2) isocalendin; (3) tagalsin Q; (4) phenaziterpene B; (5) tuberatolide A; (6) N-((2S,3S,5R)-6-(butylamino)-3-hydroxy-1-(isopropylamino)-5-methyl-6-oxohexan-2-yl)-4-methoxy-3-(3-methoxypropoxy) benzamide.

Table S2. Chemical interaction of the target compounds.

| Compound<br>(docking score)                                                    | Type of interaction | Target<br>(PDB ID) | Residue                                                                         |
|--------------------------------------------------------------------------------|---------------------|--------------------|---------------------------------------------------------------------------------|
| *Saroglitazar<br>(-9.1596)                                                     | Electrostatic bond  | PPARα<br>(6LXB)    | -                                                                               |
|                                                                                | Hydrogen bond       |                    | Ser 280, Tyr 314, His 440, His 464                                              |
|                                                                                | Π-π stacking        |                    | -                                                                               |
|                                                                                | Van Der Waals       |                    | Cys 275, Cys 276, Phe 318, Leu 321, Val 332, Ala 333, Met 355, His 440          |
| 2-[3-[5-(1-Carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid<br>(-9.4067)  | Electrostatic bond  |                    | -                                                                               |
|                                                                                | Hydrogen bond       |                    | Met 220                                                                         |
|                                                                                | Π-π stacking        |                    | -                                                                               |
|                                                                                | Van Der Waals       |                    | Met 220, Cys 275, Cys 276, Thr 279, Leu 321, Val 332                            |
| *Lanifibranor<br>(-9.90518)                                                    | Electrostatic bond  | PPARδ<br>(8HUL)    | -                                                                               |
|                                                                                | Hydrogen bond       |                    | His 287, Tyr 437                                                                |
|                                                                                | Π-π stacking        |                    | -                                                                               |
|                                                                                | Van Der Waals       |                    | Phe 246, Cys 249, Thr 252, Leu 294, Leu 303, Ile 327, Ile 328                   |
| 2-[3-[5-(1-Carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid<br>(-9.92129) | Electrostatic bond  |                    | -                                                                               |
|                                                                                | Hydrogen bond       |                    | Ala 306                                                                         |
|                                                                                | Π-π stacking        |                    | -                                                                               |
|                                                                                | Van Der Waals       |                    | Cys 249, Thr 252, Leu 294, Leu 303, Ile 327                                     |
| *Seladelpar<br>(-8.99944)                                                      | Electrostatic bond  | PPARγ<br>(8HUP)    | -                                                                               |
|                                                                                | Hydrogen bond       |                    | Ser 342                                                                         |
|                                                                                | Π-π stacking        |                    | Phe 264                                                                         |
|                                                                                | Van Der Waals       |                    | Phe 264, Gly 284, Cys 285, Arg 288, Leu 330, Ile 341                            |
| Phenaziterpene B<br>(-9.96555)                                                 | Electrostatic bond  |                    | -                                                                               |
|                                                                                | Hydrogen bond       |                    | Glu 343                                                                         |
|                                                                                | Π-π stacking        |                    | -                                                                               |
|                                                                                | Van Der Waals       |                    | Glu 259, Phe 264, His 266, Ile 281, Gly 284, Arg 288, Ile 341, Ser 342, Leu 333 |
| 2-[3-[5-(1-Carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid<br>(-9.24288) | Electrostatic bond  |                    | Arg 288                                                                         |
|                                                                                | Hydrogen bond       |                    | Arg 288, Ser 289                                                                |
|                                                                                | Π-π stacking        |                    | Phe 282                                                                         |
|                                                                                | Van Der Waals       |                    | Cys 285, Arg 288, Ser 289, Ala 292, Ile 326, Leu 330                            |

**Table S2.** Chemical interaction of the target compounds (continued...)

| Compound<br>(docking score)                                                                         | Type of interaction | Target<br>(PDB ID)           | Residue                                                         |
|-----------------------------------------------------------------------------------------------------|---------------------|------------------------------|-----------------------------------------------------------------|
| *CCR2-RA- [R]<br>(-11.04719)                                                                        | Electrostatic bond  | CCR2<br>(5T1A<br>allosteric) | -                                                               |
|                                                                                                     | Hydrogen bond       |                              | -                                                               |
|                                                                                                     | Π-π stacking        |                              | -                                                               |
|                                                                                                     | Van Der Waals       |                              | Leu 81, <b>Phe 312, Val 244</b>                                 |
| 2-[3-[5-(1-Carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid<br>(-10.065)                       | Electrostatic bond  |                              | Arg 138                                                         |
|                                                                                                     | Hydrogen bond       |                              | Arg 138, Phe 312                                                |
|                                                                                                     | Π-π stacking        |                              | Tyr 305, Phe 312                                                |
|                                                                                                     | Van Der Waals       |                              | Arg 138, Ala 241, <b>Val 244</b> , Ile 245, <b>Phe 312</b>      |
| *Compound 1 or [2-[2-oxidanylidene-2-(2-phenylethylamino)ethoxy]phenyl]phosphonic acid<br>(-7.7781) | Electrostatic bond  | ChREBP<br>(8BTQ)             | <b>Arg 128B, Arg 129A, Arg 56A</b>                              |
|                                                                                                     | Hydrogen bond       |                              | Tyr 130A, <b>Arg 128B, Arg 129A, Arg 56A</b>                    |
|                                                                                                     | Π-π stacking        |                              | <b>Trp 127B</b> , Arg 56A                                       |
|                                                                                                     | Van Der Waals       |                              | <b>Trp 127B</b> , Leu 222A                                      |
| 2-[3-[5-(1-Carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid<br>(-8.07249)                      | Electrostatic bond  |                              | <b>Arg 128B, Arg 129A, Arg 56A</b>                              |
|                                                                                                     | Hydrogen bond       |                              | <b>Arg 128B, Arg 129A, Arg 56A</b>                              |
|                                                                                                     | Π-π stacking        |                              | <b>Trp 127B</b>                                                 |
|                                                                                                     | Van Der Waals       |                              | <b>Trp 127B</b> , Asn 123B                                      |
| Tagalsin Q<br>(-7.8297)                                                                             | Electrostatic bond  |                              | -                                                               |
|                                                                                                     | Hydrogen bond       |                              | <b>Arg 56A</b> , Arg 129A                                       |
|                                                                                                     | Π-π stacking        |                              | -                                                               |
|                                                                                                     | Van Der Waals       |                              | Lys 49A, <b>Trp 127B</b>                                        |
| *Empagliflozin<br>(-11.86976)                                                                       | Electrostatic bond  | SGLT2<br>(7VSI)              | -                                                               |
|                                                                                                     | Hydrogen bond       |                              | Asn 75, Phe 98, Ser 287, Gln 475                                |
|                                                                                                     | Π-π stacking        |                              | <b>Phe 98</b> , His 80                                          |
|                                                                                                     | Van Der Waals       |                              | Val 95, <b>Phe 98</b> , His 80, Leu 84, Phe 453, <b>Tyr 290</b> |
| 2-[3-[5-(1-Carboxyethyl)thiophene-2-carbonyl]phenyl]benzoic acid<br>(-11.3459)                      | Electrostatic bond  |                              | -                                                               |
|                                                                                                     | Hydrogen bond       |                              | Thr 87                                                          |
|                                                                                                     | Π-π stacking        |                              | <b>Phe 98</b> , Tyr 290                                         |
|                                                                                                     | Van Der Waals       |                              | <b>Phe 98, Tyr 290</b>                                          |

\*Native ligand. PPAR: peroxisome proliferator-activated receptor; CCR: C-C chemokine receptor; ChREBP: carbohydrate response element-binding protein; SGLT2, sodium-glucose cotransporter-2.

**Table S3.** Calculated ΔG based on the MM/GBSA and MM/PBSA approaches for five different conformers.

| Compound            | ΔG MM/GBSA (kcal/mol) | ΔG MM/PBSA (kcal/mol) |
|---------------------|-----------------------|-----------------------|
| Saroglitasar        | -65.73 ± 3.89         | -63.36 ± 6.20         |
| Compound 1-1        | -24.75 ± 3.80         | 50.38 ± 21.94         |
| <b>Compound 1-2</b> | <b>-47.22 ± 3.19</b>  | <b>-40.94 ± 6.47</b>  |
| Compound 1-3        | -23.82 ± 3.48         | 21.02 ± 9.97          |
| Compound 1-4        | -24.39 ± 3.77         | -14.56 ± 4.06         |
| Compound 1-5        | -31.81 ± 2.78         | -15.98 ± 4.17         |

ΔG: Gibbs free energy; MM/GBSA: Molecular Mechanics Generalized Born Surface Area; MM/PBSA: Molecular Mechanics Poisson-Boltzmann Surface Area.