

Research article

Modulation of the SDF-1/CXCR4 Axis by *Tithonia diversifolia*, *Moringa oleifera* and *Curcuma longa* Extracts in Diabetic Fatty Liver

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Abstract

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder often linked to non-alcoholic fatty liver disease (NAFLD). Liver inflammation in T2DM is associated with the activation of stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4. This study aimed to evaluate the effect of herbal combinations of *Tithonia diversifolia*, *Moringa oleifera*, and *Curcuma longa* (TMC) on SDF-1 expression in a T2DM-induced fatty liver mice model and assess their potential as a CXCR4 inhibitors through *in silico* analysis. The *in silico* study involved LC-HRMS compound identification, bioactivity prediction, drug-likeness screening, molecular docking, and molecular dynamics simulations to analyze interactions with CXCR4. In the *in vivo* study, T2DM was induced by a high-fat diet and streptozotocin, followed by 21 days of oral TMC extract administration to evaluate its therapeutic effects. LC-HRMS analysis identified 23 bioactive compounds in TMC extracts, including curcumin, which after screening was used in molecular docking. Curcumin showed the strongest binding to CXCR4 (-6.3 kcal/mol), with residue similarity to IT1t and metformin. The stability of the CXCR4-Curcumin was rigorously confirmed by a 20 ns molecular dynamics simulation. SDF-1 expression was significantly increased in T2DM mice ($38.72 \pm 6.12\%$) compared to normal ($24.21 \pm 4.80\%$). The combination of TMC extracts, especially TMC3, reduced the expression to $24.59 \pm 4.68\%$ which was close to the normal group. This decrease was attributed to the polyphenol and sesquiterpene content, which acted through anti-inflammatory pathways. These results suggest that the TMC herbal combinations have potential as an alternative phytotherapeutic agent acting to inhibit SDF-1 and CXCR4 activation in T2DM-induced fatty liver.

Keywords: *Curcuma longa*; fatty liver; *Moringa oleifera*; SDF-1/CXCR4 axis; *Tithonia diversifolia*; type 2 diabetes mellitus

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1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease frequently leading to widespread health complications including those affecting the liver. A frequent liver complication in T2DM patients is non-alcoholic fatty liver disease (NAFLD) (Antar et al., 2023). The progression of NAFLD starts from simple steatosis and can escalate to nonalcoholic steatohepatitis (NASH), potentially culminating in liver fibrosis, cirrhosis, or liver cancer (Chen & Yeh, 2021). The transition from NAFLD to NASH is primarily fueled by excessive fat consumption, which leads to hyperglycemia and hyperlipidemia. These metabolic disturbances encourage the overexpression of adipocytokines and chemokines, intensifying insulin resistance in both adipose tissue and the liver. This heightened insulin resistance then triggers the liver to synthesize an abundance of triglycerides (TG) and accumulate free fatty acids (FFA) within the organ. Fat accumulation in the liver, or hepatic steatosis, is considered the first hit. Thereafter, a second hit occurs where inflammatory mediators trigger liver damage. Excessive TG accumulation triggers various harmful effects on hepatocytes, such as uncontrolled lipid peroxidation, oxidative stress, and endoplasmic reticulum (ER) stress that further activate inflammatory pathways in the liver (Xu et al., 2015; Liu et al., 2024a).

The liver serves as a vital endocrine organ that plays a role in the secretion of various cytokines and has a variety of cytokine receptors. One important type of cytokine is chemokines, which are small signaling proteins that are secreted and interact with G protein-coupled receptors (GPCRs) on target cells (Liu et al., 2024b). Chemokines are broadly classified as either homeostatic or inflammatory. Homeostatic chemokines are responsible for maintaining the normal function of the body, contributing to organ development, immune cell circulation, and stem cell migration. In contrast, inflammatory chemokines recruit immune cells to sites of inflammation or infection (Chen et al., 2018). Stromal cell-derived factor 1 (SDF-1), or CXCL-12, is a CXC chemokine found widely throughout the body, produced by various cell types including immune, endothelial, and stem cells. Specifically in the liver, biliary epithelial cells, hepatic stellate cells, and hepatic sinusoid endothelial cells generate SDF-1 (Liepelt & Tacke, 2016). SDF-1 primarily interacts with its major receptor, CXCR4, a G protein-coupled receptor found across liver tissue and on primary hepatocytes (Bianchi & Mezzapelle, 2020). Importantly, increased SDF-1/CXCR4 activity has been linked to the progression of liver disease in T2DM (Kurita et al., 2019; Liu et al., 2022).

Metformin as a first-line oral drug is commonly used in diabetes management to lower blood glucose levels (Shurrah & Arafa, 2020). However, research continues into new therapies that carry fewer side effects. Herbal medicines, with their array of bioactive compounds, are being increasingly investigated for their potential in treating metabolic diseases, building on their long-standing use in traditional medicine (Tran et al., 2020). Herbal combinations containing *Tithonia diversifolia*, *Moringa oleifera*, and *Curcuma longa* (TMC) offer exciting prospects. *T. diversifolia* is known for its antidiabetic and anti-inflammatory properties (Chunudom et al., 2020). *Moringa oleifera* provides significant anti-inflammatory, antioxidant, and hepatoprotective actions (Pareek et al., 2023). Meanwhile, *C. longa* acts as an anti-inflammatory and antioxidant agent, demonstrably effective in mitigating liver steatosis and inflammation (Fuloria et al., 2022).

The primary goal of this study is to investigate the potential inhibition of CXCR4 receptor by active compounds from TMC herbal extracts in silico through molecular docking and molecular dynamics. The effect of TMC extract combination on SDF-1 expression in fatty liver caused by T2DM was also examined. Ultimately, these findings are

expected to lay a scientific foundation for developing new plant-based therapies that target the chemokine pathway in liver complications linked to diabetes.

2. Materials and Methods

2.1 Extraction of TMC

Tithonia diversifolia leaf powder, *Moringa oleifera* leaf powder, and *Curcuma longa* rhizome powder were obtained from Materia Medica Batu, Malang, Indonesia. All plant materials were extracted using distilled water (aqueous extraction). A 100 g sample of each plant powder was weighed and then extracted with 1000 mL of boiling water (ratio 1:10). Each extract was then stirred, cooled, filtered with a filter cloth, and filtrates were evaporated to obtain a liquid extract. Each liquid extract was stored in a freezer at -80°C. Each extract was then freeze dried to obtain dry extracts (Salsabila et al., 2025).

2.2 LC-HRMS analysis

LC-HRMS analysis of TMC extracts was performed using Vanquish™ UHPLC Binary Pump liquid chromatography (Thermo Scientific™, Germany) and Q Exactive™ Hybrid Quadrupole-Orbitrap™ High Resolution Mass Spectrometer (Thermo Scientific™, Germany). Liquid chromatography column used for the analysis was a 100 mm × 2.1 mm ID × 2.6 µm Accucore™ Phenyl-Hexyl column (Thermo Scientific™, Germany). Prepared samples were then placed on an autosampler and injected into the LC-HRMS system. Data were processed using Compound Discoverer™ 3.2. The compounds displayed were compounds were identified with the mzCloud™ and ChempSpider™ database (Windarsih et al., 2022).

2.3 Data mining, screening bioactivity and drug-likeness of compounds

Compounds from the TMC aqueous extract were selected based on LC-HRMS data analysis, with their 3D structures and canonical SMILES acquired from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). Bioactivity predictions, focusing on the probable activity (Pa) values, were made using PASS Online Way2Drug (<http://www.way2drug.com/passonline>). Identified compounds were determined for drug similarity using SWISS ADME web server (<http://www.swissadme.ch/>). CXCR4 receptor was sourced from RCSB Protein Data Bank (<https://www.rcsb.org/structure/4RWS>) with PDB ID: 4RWS. Two ligand controls were used to compare with compound and protein interactions. The control in the form of native ligand IT1t was used to inhibit CXCR4 with Pubchem ID: 25147749. Control drug metformin was obtained from Pubchem ID: 4091.

2.4 Molecular docking

Molecular docking was simulated using Autodock Vina on PyRx 0.9.5 software. Prior to docking, the receptor was prepared in Biovia Discovery Studio 2019 by removing water molecules and existing ligands. All ligands were also prepared by minimizing their energy using Open Babel, integrated into PyRx. This molecular docking used specific docking. The grid position was determined in previous studies at grid center 97.4982 (X), -0.0632 (Y), 37.2387 (Z), with dimensions 13.3055 Å (X), 5.6212 Å (Y), 8.457 Å (Z) (Sa'adah et al., 2025). Validation involved five redocking replicates. Protein and ligand complexes were

combined using PyMol software and the interaction was visualized using Biovia Discovery Studio 2019 software.

2.5 Molecular dynamics

Compounds with the best binding affinity values were selected for molecular dynamics simulations. These simulations were run using YASARA (Yet Another Scientific Artificial Reality Application) version 19.12.14 software, employing the AMBER14 force field. The system was set to 37°C, pH 7.4, 0.9% salt content, 1 atm pressure, and a water density of 0.997 g/ml. Each simulation ran for 20 ns, with data automatically saved every 25 ps using the md_runfast and md_bindingenergy macro programs. Subsequent analysis involved the md_analyze program for Root Mean Square Deviation (RMSD), md_analyzeres for Root Mean Square Fluctuation (RMSF), and md_bindingenergy to determine the receptor-ligand binding energy, which was computed via the MM-PBSA method.

2.6 Induction of T2DM using streptozotocin and high-fat diet

A total of 24 male Balb/C mice aged 7-8 weeks with body weight around 20-30 g were obtained from Kemuning, Karanganyar, Indonesia. The T2DM induction model uses a high-fat diet (HFD) and multiple low doses of streptozotocin (STZ) to produce a stable T2DM phenotype, characterized by hyperglycemia, dyslipidemia, and fatty liver. This model has been widely accepted and is considered physiologically relevant for diabetes research related to metabolism and the liver. Acclimation was carried out for 14 days by feeding mice with a high-fat diet (HFD), except for the normal group. Mice were injected with streptozotocin (bioWORLD, bioPLUS™, USA) dissolved in 0.1 M citrate buffer and at pH 4.5 intraperitoneally at a dose of 50 mg/kg BW for 4 consecutive days. Blood glucose levels were measured after 5 days of STZ injection. The mice were then given 10% sucrose, and T2DM diagnosis was confirmed by their blood sugar being ≥ 200 mg/dL. Lipid accumulation, characteristic of fatty liver, was confirmed by histological analysis. The mice were divided into six groups with each group having 4 replicates, namely; (i) normal; (ii) T2DM; (iii) Metformin (150 mg/kg BW); (iv) TMC1 (*T. diversifolia* = 500 mg/kg BW, *M. oleifera* = 300 mg/kg BW, *C. longa* = 200 mg/kg BW); (v) TMC2 (*T. diversifolia* = 200 mg/kg BW, *M. oleifera* = 500 mg/kg BW, *C. longa* = 300 mg/kg BW); and (vi) TMC3 (*T. diversifolia* = 300 mg/kg BW, *M. oleifera* = 200 mg/kg BW, *C. longa* = 500 mg/kg BW). TMC extracts and metformin were dissolved in distilled water before being administered orally to rats for 21 days according to the treatment group dosage. Daily consumption of a HFD and 10% sucrose solution was maintained during the treatment period, during which body weight and glucose levels were monitored every three days. This experimental protocol was approved by the ethics committee of Universitas Brawijaya (No. 141-KEP-UB-2024).

2.7 Liver isolation and immunostaining

The mice were sacrificed and then dissected to isolate the liver. The liver was washed with phosphate buffer saline (PBS) (Thermo Fisher Scientific, USA). It was then homogenized in PBS within a petri dish, and the resulting cell suspension was transferred to a propylene tube for centrifugation (2500 rpm, 10°C, 5 min). The supernatant was discarded to obtain a pellet, then resuspended with 1 mL of sterile PBS. This cell suspension was then aliquoted into 1.5 mL microtubes, each containing 400 μ L PBS, and centrifuged again under the same conditions. The resulting hepatocyte-containing pellet was fixed using

fixation buffer (BioLegend, USA) (20 min incubation), followed by an intracellular staining perm wash buffer (BioLegend, USA) incubation (5 min) and another centrifugation. After discarding the supernatant, the final pellet was treated with PerCP anti-human/mouse CXCL12/SDF-1 intracellular antibody (IC350C, Bio-Techne, USA) for 20 min. The stained cells were resuspended in PBS and transferred to a cuvette for flow cytometry analysis (BD FACSCalibur, USA). Therefore, the flow cytometry data presented reflects the level of intracellular SDF-1 expression in liver cells.

2.8 Data analysis

The flow cytometry results were analyzed using the BD Cellquest Pro™ program. Statistical analysis of flow cytometry was performed using the SPSS program with one way of variance analysis (ANOVA) test and followed by Tukey's Post hoc test with a p-value of 0.05.

3. Results and Discussion

3.1 Bioactive compounds of TMC extracts based on LC-HRMS analysis

Analysis of the bioactive compounds in TMC extracts using LC-HRMS allowed identification and characterization of compounds based on molecular weight, chemical formula, retention time, and comparison with comprehensive mass spectrum databases such as mzCloud. The identification results are presented in Table 1, showing the presence of various compounds of diverse bioactive potential.

In total, 23 bioactive compounds were present in the TMC extract. These compounds included various classes of secondary metabolites, which are often known to have pharmacological activity. The degree of match between the candidate structure and the actual compound was determined with mzCloud (mzCloud Best Match). A higher mzCloud best match value indicates higher accuracy of the identification results where the value of most compounds was very high, often well above the benchmark which was $\geq 70\%$ (Yuan et al., 2024). The compounds with the highest mzCloud were ilicic acid and caffeic acid, which even reached 99.9%. Retention time (RT) and area (max) also provide initial quantitative information regarding the relative presence of each compound in the sample. RT refers to the duration a compound spends traveling through the column before arriving at the mass detector. Compounds with larger areas indicate relatively higher concentrations in the analyzed sample (Zang et al., 2023). Molecules with low molecular weight penetrate cell membranes more easily than larger molecules. Molecules with molecular weights below 500 Daltons (Da) tend to have better membrane permeability (Yang & Hinner, 2015). Due to its large molecular weight, rutin struggles to cross membranes, whereas suberic acid with smaller molecular weight allows for easier membrane penetration.

3.2 Prediction of bioactivity and drug-likeness

The potential bioactivity of the compounds contained in TMC extracts identified through LC-HRMS analysis was predicted using PASS online. PASS online was developed to predict the bioactivity of drug-like organic molecules, which is represented as probable activity (Pa) and probable inactivity (Pi) (Rudik et al., 2019). Figure 1A presents the bioactivity prediction results of TMC extract compounds, where a greater degree of purple color indicates a higher probability of the compound having biological activity. If the value

of $P_a > 0.7$, the probability of bioactivity of the compound is high, (ii) if $0.3 < P_a < 0.7$, the bioactivity of the compound is moderate, and (iii) $P_a < 0.3$, the bioactivity of the compound is low (Filimonov et al., 2014). It can be seen that the compounds contained in TMC extracts have a high probability of bioactivity in almost all parameters, making it a promising approach to overcome liver disease due to T2DM.

Table 1. TMC bioactive compounds based on LC-HRMS analysis

No	Compound Name	Formula	Molecular Weight (g/mol)	RT (min)	Area (max)	mzCloud Best Match
1	(±)9-HpODE	C ₁₈ H ₃₂ O ₄	312.23	10.641	14270329.69	71.3
2	(±)-Absciscic acid	C ₁₅ H ₂₀ O ₄	264.14	7.501	5996548.674	90.9
3	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	C ₁₈ H ₃₄ O ₅	330.24	8.922	164437871.2	86.2
4	4-Feruloylquinic acid	C ₁₇ H ₂₀ O ₉	368.11	5.013	56467182.31	99.3
5	Illicic acid	C ₁₅ H ₂₄ O ₃	252.17	9.837	99613159.79	99.9
6	4,5-Dicaffeoylquinic acid	C ₂₅ H ₂₄ O ₁₂	516.13	6.213	98328855.32	94
7	Kaempferol-3-O-rutinoside	C ₂₇ H ₃₀ O ₁₅	594.16	5.977	41698786.03	96.9
8	Azelaic acid	C ₉ H ₁₆ O ₄	188.1	6.479	83938952.85	99.3
9	Caffeic acid	C ₉ H ₈ O ₄	180.04	4.078	22879608.71	99.9
10	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	354.09	3.818	865282002.8	99.6
11	Citric acid	C ₆ H ₈ O ₇	192.03	0.981	1831028484	99.5
12	Corchorifatty acid F	C ₁₈ H ₃₂ O ₅	328.22	8.511	246449021.9	98.8
13	Curcumin	C ₂₁ H ₂₀ O ₆	368.13	10.96	16712362.4	95.5
14	D-(-)-Fructose	C ₆ H ₁₂ O ₆	180.06	0.779	75046273.72	75.7
15	D-(-)-Quinic acid	C ₇ H ₁₂ O ₆	192.06	3.7	58122013.4	88
16	Dodecanedioic acid	C ₁₂ H ₂₂ O ₄	230.15	6.458	12213929.05	96.8
17	Ferulic acid	C ₁₀ H ₁₀ O ₄	194.06	5.725	9961018.015	98.5
18	Gluconic acid	C ₆ H ₁₂ O ₇	196.06	0.767	371901433.9	97.2
19	Neochlorogenic acid	C ₁₆ H ₁₈ O ₉	354.09	2.096	910524632.4	99.4
20	Quercetin-3β-D-glucoside	C ₂₁ H ₂₀ O ₁₂	464.1	5.734	32888776.14	98.8
21	Rutin	C ₂₇ H ₃₀ O ₁₆	610.15	5.529	255996846.1	96.1
22	Suberic acid	C ₈ H ₁₄ O ₄	174.09	5.318	7085747.238	97.8
23	α,α-Trehalose	C ₁₂ H ₂₂ O ₁₁	342.12	0.775	202208341.5	98.2

Drug-likeness is an important concept in drug discovery that is used to evaluate whether a compound has characteristics that resemble those of successfully developed drugs (Li et al., 2024). In general, drug-likeness is based on the similarity of a compound's physicochemical properties or chemical structure with approved drugs. By conducting an initial screening based on this criterion, compounds that have a high risk of side effects or low efficacy can be eliminated before moving on to further development (Lee et al., 2022). Drug-likeness selection on TMC extract compounds was based on five parameters, namely Lipinski, Ghose, Veber, Egan, and Muegge for more accurate virtual drug-likeness selection (Figure 1B).

Lipinski's Rule of Five (RO5) is based on compounds having a molecular weight not exceeding 500 Da, MLOGP value not greater than 4.15, hydrogen bond acceptors not more than 10, and a maximum of 5 hydrogen bond donors. The Ghose filter suggests an optimal drug candidate should have a MW of 160-480 Da, a WLOGP ranging from -0.4 to 5.6, a molar refraction around 40-130, and an atomic number of 20 to 70. Veber's rule has the criteria that a compound should have rotatable bonds less than 10 and TPSA less than 140 Å². For effective oral absorption, the Egan filter states that a compound should have a WLOGP of less than 5.88 and TPSA not exceeding 131 Å². Muegge's rule strictly calls for the selection of drug-like compounds with MW around 200-600 Da, XLOGP3 between -2 and 5, TPSA less than 150 Å², the number of rings less than 7, a maximum of 4 carbon atoms, heteroatoms more than 1, a maximum of 15 number of rotatable bonds, hydrogen bond acceptors less than 10, and hydrogen bond donors less than 5 (Pathania & Singh, 2021). Overall, two compounds were obtained that passed the drug-likeness selection that did not appear to have violations in the five parameters, namely ilicic acid and curcumin. The two compounds were then used in molecular docking analysis.

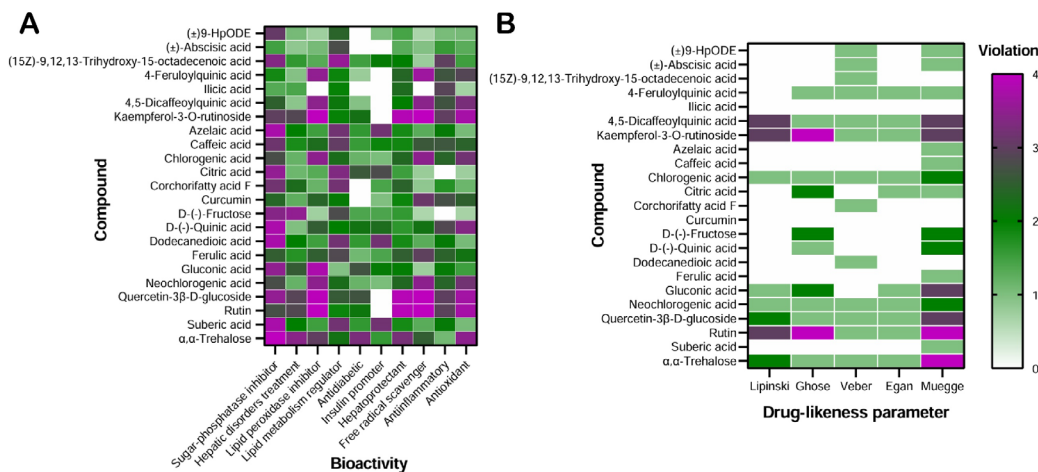


Figure 1. Predicted compounds in TMC extracts. (A) bioactivity and (B) drug-likeness

3.3 Molecular docking

Molecular docking is used to predict how a compound interacts with its molecular target. This analysis is designed as an aid to drug discovery and development (Pinzi & Rastelli, 2019). Molecular docking in this study uses CXCR4 as the receptor. The ligands that interacted with CXCR4 were ilicic acid and curcumin from TMC extracts that had passed

drug-likeness screening, IT1t was the control, and metformin was the antidiabetic drug control. IT1t was chosen as the control because it belongs to a group of isothiourea compounds that function as CXCR4 receptor antagonists and show high inhibitory potential with excellent bioavailability and in vivo activity (Dekkers et al., 2023). Metformin, a common T2DM treatment, works by influencing several cellular pathways in the liver, gut, and muscle. Its mechanism involves counteracting insulin resistance, lowering blood glucose, improving lipid metabolism, and mitigating inflammation (Bailey, 2024).

The binding affinity of -6.9 kcal/mol indicated that IT1t had a very strong and stable interaction with the CXCR4 binding site, thus proving its role as an effective antagonist (Figure 2A). This value is an important reference for evaluating the potential of other ligands. Metformin has the highest binding affinity (-4.5 kcal/mol) when binding to CXCR4 compared to other ligands. Although the binding ability of metformin was higher than the IT1t control, this value indicated that metformin could still interact with CXCR4 weakly. Illicic acid demonstrated a notable binding affinity of -6.0 kcal/mol with CXCR4. However, curcumin exhibited a particularly strong interaction, achieving the lowest binding affinity value of -6.3 kcal/mol. This value closely rivalled that of the IT1t control and surpassed both illicic acid and metformin, which were used as drug controls. Binding affinity quantifies the strength of a receptor-ligand interaction, with lower values indicating a stronger bond and more precise molecular docking predictions (Daggupati et al., 2017).

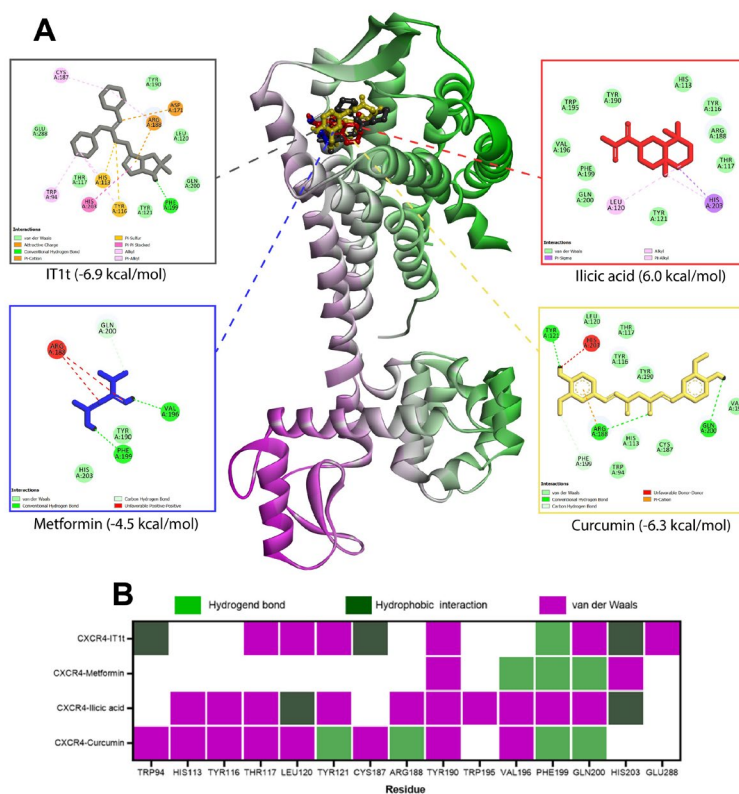


Figure 2. Molecular docking of potential compounds with CXCR4. (A) Visualization of molecular docking and (B) Amino acid residue position on chemical interaction

The molecular docking analysis, shown in Figure 2B, further indicates the analogous amino acid residues involved in receptor-ligand binding. IT1t binds tightly and specifically to the active site of CXCR4 through hydrophobic interactions, hydrogen bonding, and van der Waals forces involving most of the key residues. Metformin exhibits hydrogen bonding with amino acid residue PHE199 as well as van der Waals with amino acid residues TYR190 and GLN200 in common with IT1t. Ilicic acid formed hydrophobic interactions with HIS203 and van der Waals with THR117, TYR121, TYR190, and GLN200 which were the same as IT1t. Molecular docking showed that curcumin interacts with CXCR4 through various forces. Specifically, it formed hydrogen bonds with PHE199 (similar to IT1t and metformin) and GLN200 (similar to metformin). Van der Waals forces were also noted with THR117, LEU120, and TYR190. Hydrogen bonding plays an important role in controlling the stability of the interaction between the receptor complex and the ligand (Vaidyanathan et al., 2023). Hydrophobic interactions are considered the main driving force for ligands binding to proteins (Liu et al., 2024b). Van der Waals forces contribute to the precise fit and specificity between the ligand and receptor by minimizing unfavorable interactions (Tallei et al., 2025). Previous studies reported that the amino acid residue HIS203 is one of the main roles in the interaction with CXCR4 (Smaldone et al., 2025). In addition, other important residues in CXCR4 are Asp187, Asp97, Asp262, and Glu288 (Wahono et al., 2024). Given its superior binding affinity from the molecular docking results, curcumin was selected for further molecular dynamics analysis.

3.4 Molecular dynamics

Molecular dynamics is a computer simulation that studies biological molecules by visualizing the movement of their atoms and molecules. This method mimics the structural evolution of biological molecules over time, thereby providing detailed atomic-level understanding of these changes (Badar et al., 2022). Figure 3 displays a series of graphs of molecular dynamic simulation results over 20 ns for protein-ligand complexes involving curcumin, metformin, and IT1t.

RMSD Backbone (RMSD Bb) measures the deviation of the protein's main skeleton building atoms (generally N, C α , C) from the initial structure throughout the simulation. In other words, this parameter reflects the structural stability of the protein (Aier et al., 2016; Pang et al., 2021). The RMSD Bb of all complexes fluctuated as the duration of simulation time increased. The CXCR4-IT1t complex showed a drastic increase to about 11-14 Å at the end of the simulation indicating instability. The CXCR4-Metformin complex experienced a gradual fluctuation of RMSD Bb from the beginning of the simulation with the highest fluctuation of about 11 Å at 16 ns and then dropped to 5 Å at the end of the simulation. The CXCR4-Curcumin complex had a similar RMSD Bb to the CXCR4-Metformin complex, although its value increased at 10 ns to reach 10 Å (Figure 3A).

RMSD Ligand Movement (RMSD LigMov) was used to measure the displacement of the ligand position in the protein binding pocket during simulation. A low ligand RMSD indicates that the ligand is firmly and stably bound within the binding pocket of the receptor structure and movement is minimal (Halder et al., 2023). The RMSD LigMov for CXCR4-IT1t was more stable from the beginning to the end of the simulation than those of other complexes. The CXCR4-Curcumin complex also had similar stability to the CXCR4-IT1t complex, although it increased to 8 Å at 6 ns and 9 Å at 15-16 ns. Meanwhile, the CXCR4-Metformin complex showed considerable fluctuations compared to the other complexes, reaching around 11-15 Å at 5 ns, 10 ns, and 17 ns (Figure 3B).

RMSD Ligand Conformation (RMSD LigConf) measures the change in ligand conformation during simulation. A stable conformation indicates that the ligand is in a strongly bound state, whereas an unstable conformation indicates high flexibility (Hirano et al., 2021). The LigConf RMSD was relatively stable across complexes compared to other RMSDs. The CXCR4-IT1t complex was relatively stable with fluctuations of about 2-3 Å. CXCR4-Metformin showed very high conformational stability with fluctuations below 2 Å from the beginning to the end of the simulation. The CXCR4-Curcumin complex tended to be stable around 2 Å although it demonstrated fluctuations at 4 ns, 6 ns, 15-17 ns reaching 4 Å (Figure 3C).

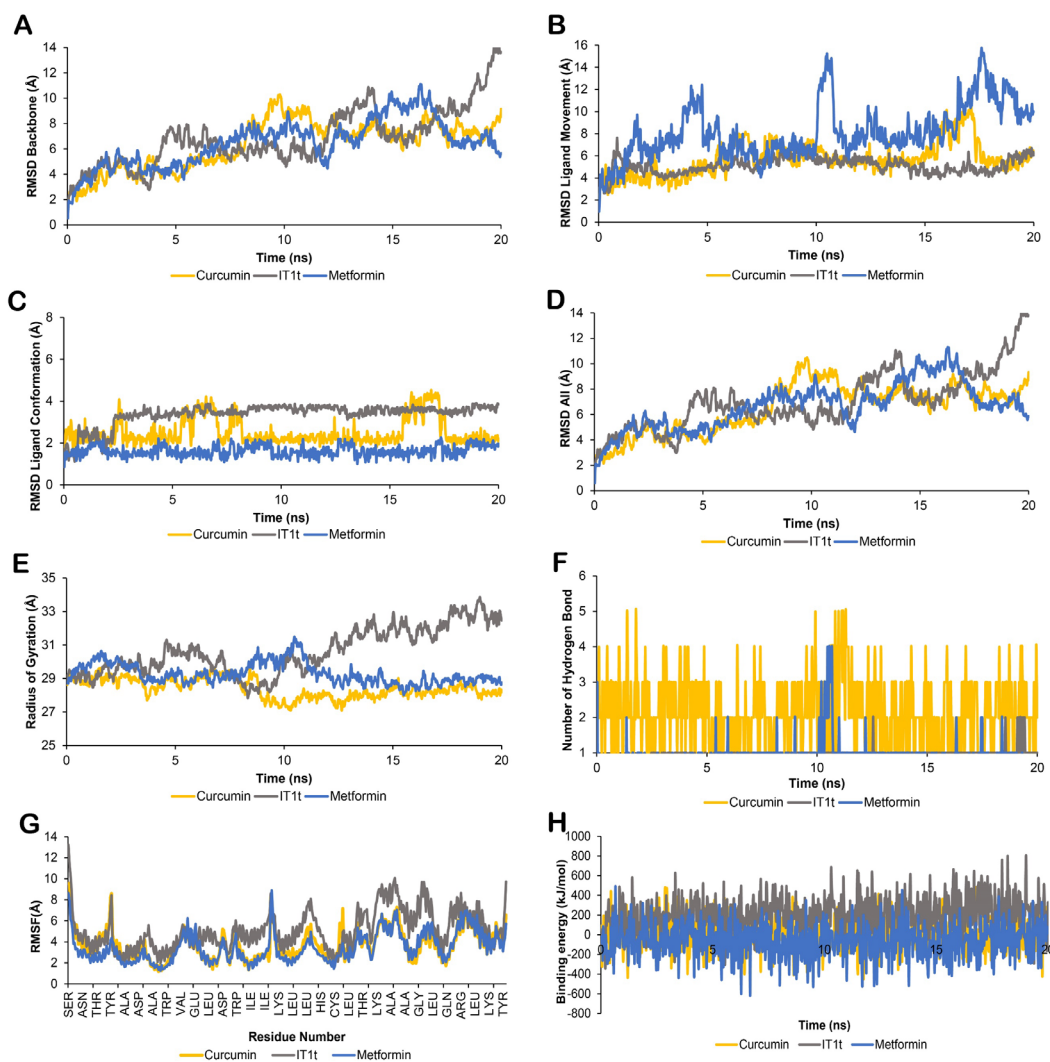


Figure 3. Molecular dynamics of potential compounds with CXCR4. (A) RMSD Backbone, (B) RMSD Ligand Movement, (C) RMSD Ligand Conformation, (D) RMSD All, (E) Radius of Gyration, (F) Number of hydrogen bonds, (G) RMSF, and (H) Binding energy

Interestingly, the RMSD All in this molecular dynamic simulation displays similar results to the RMSD of Bb. CXCR4-Metformin and CXCR4-Curcumin complexes tended to be more stable compared to the CXCR4-IT1t complex (Figure 3D). Radius of Gyration (Rg) is a measure of the degree of compactness of a protein structure during simulation. A low Rg indicates a more compact and well-folded structure (Ahmed et al., 2020). The Rg for the CXCR4-IT1t complex showed a significant increase over time, reaching around 31-33 Å. The Rg for the CXCR4-Metformin complex was relatively stable, around 28-30 Å. The CXCR4-Curcumin complex showed the most stable and compact Rg, ranging from 27-29 Å (Figure 3E).

The number of hydrogen bonds plays an important role for stability and ligand binding. The more numerous and stable hydrogen bonds between a protein and ligand indicate a strong affinity (Yu et al., 2024). During the simulation, the CXCR4-IT1t complex consistently displayed the fewest hydrogen bonds, typically ranging from 0 to 1, suggesting a low level of stability and weak binding. The CXCR4-Metformin complex had a similarly low number of hydrogen bonds, during the simulation, but at 10 ns the number increased to 4 hydrogen bonds. In contrast, the CXCR4-Curcumin complex exhibited the most consistent and highest number of hydrogen bonds, maintaining a range of 2 to 5 hydrogen bonds throughout the entire simulation, indicating a more stable and robust interaction (Figure 3F).

RMSF measures the structural deviation or fluctuation at each protein residue (Alandijany et al., 2023). The CXCR4-IT1t complex showed higher fluctuations at most residues indicating instability. CXCR4-Metformin and CXCR4-Curcumin had lower RMSF values at the same residues than CXCR4-IT1t (Figure 3G). Binding energy evaluates the strength of affinity between ligand and receptor. Ligands with more negative binding energy are considered to have higher affinity (Hata et al., 2021). The CXCR4-IT1t complex had a binding energy between -300 to 800 kJ/mol which indicated the lowest affinity. The binding energy for CXCR4-Metformin ranges from -600 to 500 kJ/mol, indicating the strongest affinity. The CXCR4-Curcumin complex showed a binding energy that was not much different from that of CXCR4-Metformin, which was around -400 to 400 kJ/mol (Figure 3H).

The molecular dynamics simulation results showed that the CXCR4-curcumin complex was quite stable compared to the two control complexes. Docking predicted that curcumin had a strong affinity and similar interaction residues to IT1t, and molecular dynamics further confirmed that these interactions remained stable over time, indicating strong and persistent binding. The combined docking and molecular dynamics evidence reinforced the conclusion that curcumin has significant potential to bind and modulate CXCR4, supporting its relevance to the *in vivo* studies observed in this research.

3.5 Effect of TMC extract on SDF-1 percentage in fatty liver of T2DM mice

Chemokines are small molecules released in tissues as part of the initial response to cell damage. Their primary role is to regulate cell migration to sites of infection or inflammation by interacting with specific G-protein-coupled receptors (GPCRs), which possess seven transmembrane domains (Tripathi & Poluri, 2020). Among these, SDF-1 is broadly distributed across body tissues and is a key player in numerous biological processes, including embryogenesis, organogenesis, regeneration, immune evasion, inflammatory responses, angiogenesis, and tumorigenesis (Liu et al., 2024a). The role of SDF-1 in inflammation is quite contradictory. It can be pro-inflammatory by drawing various immune cells, like monocytes, into fat tissue where they become inflammation-promoting cells. The interaction of SDF-1 with its receptor, CXCR4, is believed to trigger the buildup of pro-

inflammatory M1 macrophages, boosting inflammatory cytokine production and ultimately leading to insulin resistance (Kim et al., 2014). Conversely, SDF-1 also shows anti-inflammatory properties. It achieves this by guiding T cells to differentiate into regulatory T cells (Tregs), which soothe inflammation, and by stimulating M2 macrophages to produce anti-inflammatory cytokines (Karin, 2010). Furthermore, SDF-1 promotes monocyte migration and their development into the M2 phenotype, suggesting its potential to mitigate inflammation in T2DM (Vidakovic et al., 2015). Figure 4 displays the effect of TMC administration on SDF-1 expression in fatty liver of T2DM mice.

In this study, T2DM mice exhibited a significantly higher ($p < 0.05$) percentage of SDF-1 expressing cells ($38.72 \pm 6.12\%$) compared to the normal group ($24.21 \pm 4.80\%$). T2DM conditions are often caused by insulin resistance, hyperglycemia, and dyslipidemia that trigger cellular stress and inflammation. The inflammatory process intensifies due to the activation of pro-inflammatory cytokines, chemokines, and adipocytokines (Rehman & Akash, 2016). SDF-1, as a potent chemotactic chemokine, will increase under diabetic and inflammatory conditions in the liver, thereby promoting the recruitment of immune cells to inflamed hepatic tissue (Cao et al., 2021). The subsequent influx of immune cells, notably hepatic macrophages (both Kupffer cells and migrating macrophages from bone marrow), contributes to the production of inflammatory mediators, such as $\text{TNF}\alpha$, $\text{IL-1}\beta$, and reactive oxygen species (ROS). These mediators further exacerbate the inflammatory response, contributing to steatosis and fibrosis (Xu et al., 2015). Additionally, cellular damage from excessive lipid buildup triggers the release of DAMPs (damage-associated molecular patterns), which activate immune cells like macrophages, mast cells, dendritic cells, and endothelial cells. This activation stimulates the production of large amounts of various types of chemokines, one of which is SDF-1, which functions to direct the migration of neutrophils in a focused manner to the injured tissue area (Yang et al., 2025).

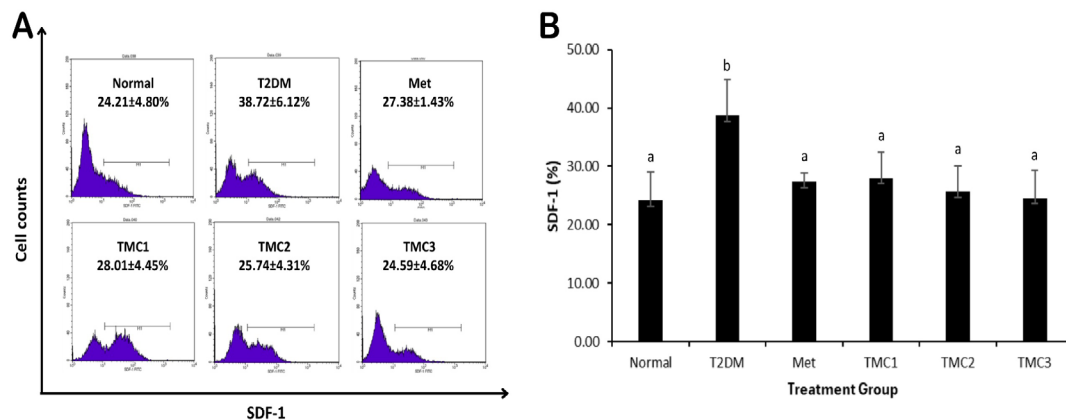


Figure 4. Effect of TMC extract on SDF-1 percentage in fatty liver of T2DM mice. (A) Flow cytometry analysis results of SDF-1 cells in the liver and (B) ANOVA test results of SDF-1 cells in liver. Lowercase letters denote significant differences ($p < 0.05$) among the treatments

In T2DM and fatty liver disease, the liver is under chronic metabolic stress and inflammation, leading to increased SDF-1 production. Increased SDF-1 then binds to CXCR4 on the surface of various liver cells. CXCR4 activation triggers several intracellular signaling pathways that directly enhance immune cell protection, pro-inflammatory cytokine production, insulin resistance, and hepatic stellate cell activation, which promote inflammation and fibrosis. SDF-1 activation of CXCR4 forms an inflammatory loop that amplifies NF- κ B and MAPK signaling, thereby increasing SDF-1 production and liver inflammation. Inhibition of CXCR4 by compounds contained in TMC extract, as predicted through docking and supported by the stability of the CXCR4–curcumin complex in molecular dynamics simulations, had the potential to break this inflammatory loop and indirectly reduce SDF-1 expression by decreasing pro-inflammatory signals. Furthermore, CXCR4 inhibition of immune cells limits the ability of SDF-1 to recruit inflammatory cells to the liver. The overall reduction in inflammation and liver stress can lead to a decrease in inflammatory signals that drive SDF-1 production.

Mice given metformin managed to reduce the percentage of cells expressing SDF-1 to $27.38 \pm 1.43\%$. This decrease shows the positive effect of metformin as an anti-inflammatory and hepatoprotective in reducing stress and inflammation in the liver induced by T2DM (Pinyopornpanish et al., 2021). Metformin counters inflammation and oxidative stress by inhibiting the creation of Advanced Glycation End Products (AGEs). When AGEs bind to their receptor, RAGE, on macrophages, the binding escalates the production of pro-inflammatory cytokines such as TNF- α , IL-1, and IL-6, and activates the NF- κ B pathway, driving inflammation, apoptosis, and fibrosis. Metformin exerts its effect by neutralizing the dicarbonyl precursors of AGEs, thus preventing their synthesis. Additionally, metformin interacts with RAGE through AMPK activation, further curtailing AGE formation. This modulation ultimately shifts macrophage phenotypes from pro-inflammatory to anti-inflammatory, consequently diminishing the intensity of the immune response (Goel et al., 2022).

A notable reduction in SDF-1 expression was observed after a 21-day treatment period with TMC extract. The effect of reducing SDF-1 by TMC1 extract was similar to metformin at $28.01 \pm 4.45\%$. TMC2 showed a decrease to $25.74 \pm 4.31\%$, which was better than TMC1 and even close to or slightly above the normal group. TMC3 extract gave the best result among TMC extracts and metformin, which was slightly lower than the normal group at $24.59 \pm 4.68\%$. These results indicate that the TMC herbal combinations, especially at the TMC3 dose, potentially has a strong anti-inflammatory effect that was comparable or even better than metformin in suppressing SDF-1 expression in T2DM-induced fatty liver conditions.

TMC extracts contain various classes of compounds based on the LC-HRMS results. Polyphenols can suppress the inflammatory response through various pathways, such as inhibiting the expression of pro-inflammatory genes, decreasing the production of inflammatory mediators, and reducing the activity of enzymes involved in the inflammatory process such as MAPK, cyclooxygenase, and lipoxygenase (Sun et al., 2024). Inflammatory mediators such as macrophages and monocytes, as well as pro-inflammatory cytokines (IL-1 β , IL-2, IL-6, IL-8, TNF α , and IFN- γ) can also be inhibited by polyphenols (Yahfoufi et al., 2018). Polyphenols that have antioxidant properties also help eliminate free radicals generated during inflammation that cause cell damage by capturing unpaired electrons and stabilizing them (Rudrapal et al., 2022). The sesquiterpene class is characterized by significant pharmacological actions, including proven anti-inflammatory, hypoglycemic, and hypolipidemic activities (Safavi et al., 2025). Sesquiterpenes are reported to exhibit diverse therapeutic activities, including α -glucosidase inhibition,

suppression of the NF- κ B pathway, AMPK agonist activity, dual PPAR α / γ agonism, and the regeneration and revitalization of residual β cells (Salazar-Gómez et al., 2020).

4. Conclusions

The TMC extracts demonstrated strong potential in reducing SDF-1 expression in a T2DM mouse model with fatty liver. The TMC3 formula normalized SDF-1 expression and demonstrated comparable effectiveness, even surpassing that of metformin. LC-HRMS analysis identified curcumin and ilicic acid as the primary compounds. These compounds showed high drug-likeness and affinity for CXCR4 based on docking and molecular dynamics simulations, with curcumin demonstrating the most stable interaction. The mechanism of SDF-1 reduction is likely related to the activity of polyphenols and sesquiterpenes, which influence inflammatory-metabolic pathways. Suggestions for further research include expanding the docking grid or using blind docking to capture additional potential interactions and conducting in vitro assays to validate the effects of TMC at the cellular level before moving on to long-term clinical studies.

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6. Authors' Contributions

Nabila Shafa Yumna Salsabila: Formal analysis, Visualization, Software, and Writing - Review & Editing.

Rifatul Hawani Burhan: Conceptualization, data analysis, and Writing - Review & Editing.

Muhaimin Rifa'i: Conceptualization, investigation, and Writing - Review & Editing.

Noviana Dwi Lestari: Conceptualization, Visualization, and Writing - Review & Editing.

Moh Dliyauddin: Conceptualization, coordinated research, methodology, validation, formal analysis, investigation, Writing - Original Draft, and Writing - Review & Editing.

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7. Conflicts of Interest

The authors declared no conflict of interest.

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