

Evaluation of Antitoxic and Anti-Inflammatory Activities of Water Extract from Mangrove *Sonneratia caseolaris* Leaves Through an *In Vitro* and *In Silico* Approaches

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Abstract

Leaves of *Sonneratia caseolaris*, traditionally used in Indonesia, contain various bioactive compounds including saponins, tannins, flavonoids, and phenolics, indicating potential as a source of anti-inflammatory agents. This study evaluated the bioactivity potential of TEMPO, a compound tentatively identified by LC-HRMS in *S. caseolaris* leaves extract, through assessed the toxicity of aqueous leaves extracts using human TIG-1 cells and *Artemia salina* larvae and *in silico* analysis. Cytotoxicity toward TIG-1 fibroblasts was assessed using cell viability assays, and the brine shrimp lethality test was used to determine LC₅₀ values of extracts prepared with different aqueous solvents. Molecular docking was performed against the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6, followed by molecular dynamics simulations to examine the stability of ligand-protein interactions. Toxicity assays showed no significant cytotoxicity, with TIG-1 cell viability remaining above 70%, while LC₅₀ values for all aqueous extracts exceeded 10,000 ppm in the brine shrimp assay. Docking analysis suggested that TEMPO may interact with overlapping binding regions of TNF- α and IL-1 β relative to reference ligands, while exhibiting a distinct predicted binding site on IL-6. Molecular dynamics simulations indicated stable interactions between TEMPO and the target cytokines throughout the simulation period. These findings indicate that the aqueous extracts showed low toxicity under the tested conditions and TEMPO exhibits potential anti-inflammatory activity. However, further *in vitro* and *in vivo* studies are needed to confirm the biological activity and safety of TEMPO for pharmaceutical applications.

Keywords: Anti-inflammatory compound, Antitoxic compound, Natural product bioactivity, Preclinical safety assessment, *Sonneratia caseolaris* leaves, Sustainable bioresource utilization

Introduction

Inflammation is a complex biological response to harmful stimuli, such as infections, injuries, or irritants, that serves to protect tissues and initiate the healing process [1-3]. Inflammation is characterized by immune system activation and the release of pro-inflammatory cytokines [2,4]. The mechanism of inflammation begins with the recognition of pathogens or tissue damage by immune cells, which then release inflammatory mediators to recruit leukocytes to the site [5]. TNF- α , IL-6, and IL-1 β have important roles in modulating the inflammatory process [6,7]. TNF- α is produced by macrophages and other immune cells, functions to increase blood vessel permeability, and to recruit immune cells to the area of inflammation [8]. IL-1 β plays a role in the activation of immune cells and increases the expression of adhesion molecules, facilitating leukocyte migration to the area of inflammation [9,10]. Meanwhile, IL-6 contributes to the inflammatory response by inducing the production of acute phase proteins in the liver and influencing T-cell differentiation [11]. Excessive production or dysregulation of these cytokines can cause tissue damage and contribute to chronic inflammatory diseases, such as autoimmune [12] and rheumatoid [13].

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids is known to have significant potential side effects [14-17]. NSAID drugs such as ibuprofen can cause gastrointestinal disorders, including abdominal pain, nausea, diarrhea, and the risk of gastrointestinal bleeding [18]. Long-term use of NSAIDs can increase the risk of impaired kidney function, edema, and hypertension [19-21]. Meanwhile, the use of corticosteroids can cause hyperglycemia, hypertension, dyslipidemia, and central obesity, which increase cardiovascular risk [22-24]. The significant side effects of these drugs highlight the need for alternative anti-inflammatory agents with fewer adverse effects. It is known that the development of natural-based products for inflammation treatment has become a significant research focus in recent years. The development of natural-based anti-inflammatory drugs not only offers a safer alternative, but is also potentially more affordable and biocompatible [8, 25].

Sonneratia caseolaris, commonly known as *pidada merah*, is a mangrove species from the Sonneratiaceae family. It typically grows between 5 - 15

meters in height and is widely distributed along the Indonesian coastline [26]. Like other types of mangroves, *S. caseolaris* also has respiratory roots and flowers with many stamens [27]. The Indonesian people have long known this type of mangrove to have various health benefits. Parts of this plant, such as bark, leaf, fruit, flowers, and seeds have significant antioxidant activity, helping to protect the body from oxidative damage associated with various degenerative diseases [28-30]. The antioxidant power is given because *S. caseolaris* contains active compounds, such as saponins, flavonoids, alkaloids, tannins, and phenols [31,32]. *Pidada merah* fruit is also rich in vitamin C, which plays an important role in boosting the immune system and also functions as a natural antioxidant. The results of other studies show that *S. caseolaris* has antibacterial activity, so it has the potential to fight bacterial infections [28,31,33], anticytotoxicity [29], antiallergic and antidiabetic [34].

Mangrove *S. caseolaris* is also known to have the potential as an anti-inflammatory agent. Recent studies have shown that *pidada merah* leaves extract has the potential as an anti-inflammatory agent. The leaf extract was effective in inhibiting inflammation in a rat model induced by carrageenan [35]. This anti-inflammatory effect is thought to be related to the content of bioactive compounds such as alkaloids, steroids, phenols, and flavonoids in the leaf. In addition, GC-MS analysis showed the presence of compounds such as aldehydes, glucosinolates, coumarins, esters, terpenoids, alcohols, lipids, tocopherols, and steroids, which contribute to anti-inflammatory activity. The presence of coumarin, for example, is known to have a variety of mechanisms of action in reducing inflammation and has the potential for treating inflammatory conditions. Other studies have shown that methanol extract of *S. caseolaris* leaves has high anti-inflammatory activity, with the percentage of edema inhibition reaching 58.24% at a concentration of 100 $\mu\text{g/mL}$ [36].

Among the compounds identified in *S. caseolaris* leaves extract, TEMPO was selected for this study because preliminary phytochemical profiling indicated it as a major constituent with predicted pharmacological potential. Previous reports have also suggested that TEMPO and structurally related compounds may exhibit antioxidant and anti-inflammatory activities through modulation of oxidative stress and

inflammatory signaling pathways. Therefore, TEMPO was considered a promising candidate for further investigation. Accordingly, this study aimed to assess the toxicity of *S. caseolaris* aqueous leaves extracts using human TIG-1 cells and *Artemia salina* larvae and to evaluate the predicted anti-inflammatory potential of TEMPO through *in silico* analysis.

Materials and methods

Sampling

Sonneratia caseolaris leaves were obtained from Ujung Pangkah Gresik, East Java (6.8899940 °S, 112.6081658 °E). The identification of *S. caseolaris* was conducted at the Laboratory of Taxonomy, Structure, and Plant Development, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, under the reference number 0291/UN10.F09.42/04/2024. Samples were taken from the third to the fifth leaves from the top of the plant. The leaves were washed from salt using water and cooled for 7 days at 25 °C. The leaves were mashed in the form of flour to be extracted.

Extraction

The water extracts used in this study included distilled water (A), and bottled drinking water from three brands: Ax (B), Cx (C), and Kx (D). Methanol (E) was used as the control. Bottle drinking water (B - D) can be found in the Indonesian market. The fluor of mangrove leaf was extracted according to the treatment (1:3, w/v), maceration at room temperature (3×24 h), and filtered with Whatman no 42. Then, filtrates were evaporated with a rotavapor at 40 °C (DLab rotavapor RG100-S).

Toxicity analysis

Toxicity analysis of mangrove leaf water extract samples *S. caseolaris* was carried out *in vitro* using TIG-1 cells. TIG-1 cells with a density of 7.5×10^3 were planted in 96 well plates. The cells were incubated for 24 h in an incubator at 37 °C and 5% CO₂. The cell medium was discarded and replaced with a treatment medium (samples A - E) with different concentrations. After 24 h, the treatment medium was discarded and 100 µL of culture medium containing MTT (0.5 mg/mL) was added to each well of the 96-well plate. The cells were incubated for 3 h, the medium containing MTT was

discarded and the formazan crystals that had formed were dissolved in 100 µL of DMSO. The plate was incubated for 30 min and the absorbance of the formazan crystals was read with a Microplate reader at a wavelength of 595 nm. The absorbance data was converted to determine the percentage of living cells using the following formula:

Cell Viability (%) =

$$\frac{\text{Sample absorbance} - \text{Blank absorbance}}{\text{Control cell absorbance} - \text{Blank absorbance}} \times 100\% \quad (1)$$

LC₅₀ analysis

An LC₅₀ testing was conducted using *Artemia salina* Leach nauplii. Hatching *Artemia* eggs with brine salt water for 48 h at room temperature. The nauplii used in this test were selected by observing under a microscope and those with agile movements. The test solution was made with concentrations of 0, 10, 100, 1,000, and 10,000 ppm. A 10 mL vial tube was filled with seawater and concentrations according to treatment. Each concentration was filled with 10 *Artemia nauplii*. Repetition was carried out 3 times. Observations were made by counting the dead *Artemia nauplii* for 24 h. The LC₅₀ value was carried out by probit analysis using the SPSS application with a 95% confidence interval.

Drug-likeness and pharmacokinetic analysis of TEMPO

The Swiss ADME web server carried out the physicochemical and pharmacokinetic prediction. The physicochemical aims to determine molecular weight, number of rotatable bonds, number of H bonds acceptor, number of donor H bonds, topological polar surface area (TPSA), lipophilicity (Consensus logP), and water solubility (LogS). The pharmacokinetic aims to determine the bioavailability score, (blood-brain barrier) BBB, Pgp substrate, Cyp inhibitor, Log-Kp, alerts, Leadlikeness violation, and Synthetic accessibility [37].

Ligand and protein preparation

TEMPO was tentatively identified in the water extract of *S. caseolaris* leaves based on LC-HRMS profiling conducted at the Central Laboratory of Life Sciences, Universitas Brawijaya. Compound annotation was performed by matching the detected mass spectral

features with reference database entries. Because no purification or structural confirmation by NMR/standard comparison was performed, TEMPO identification should be considered tentative. TEMPO compounds and inhibitors of each protein are used as ligands, aiming to determine the anti-inflammatory activity of the compound. TEMPO compound is expected to inhibit the activity of TNF- α , IL6, and IL-1 β as a pro-inflammatory agent. Ligands were obtained from the PubChem web server (<http://pubchem.ncbi.nlm.nih.gov>) in Sybil Data Files (SDF) format, then converted into 3D structures in Protein Data Bank (PDB) format with BIOVIA Discovery Studio. Receptors (TNF- α : 2AZ5; IL-6: 1P9M; and IL-1 β : 1ITB) were obtained from the RCSB PDB web server (<https://www.rcsb.org/>). Water molecules and ligands attached to the receptors were removed with BIOVIA Discovery Studio.

Molecular docking and ligand-receptor interaction analysis

Molecular docking is a ligand-receptor docking process, aimed at determining the level of ligand activation or inhibition based on the binding affinity value. Specific docking is a molecular docking method used in this study. Specific docking is a docking process carried out based on the location of the active site of each receptor [38-40]. This process uses AutoDock Vina which is integrated with PyRx version 0.9.5. Analysis of docking results, binding positions, and amino acid residues formed from the ligand-receptor interaction complex was carried out using BIOVIA Discovery Studio.

Simulation of molecular dynamic

Molecular dynamics (MD) is an analysis process that aims to determine the stability of the ligand-receptor interaction complex. This simulation was carried out using Yet Another Scientific Artificial Reality Application known as YASARA. The physiological conditions of cells (temperature of 37 °C, pH 7.4, 1 atm, and salt content of 0.9%) were used as simulation parameters. The simulation was carried out during 50 ns with autosaved every 25 ps. The simulation was run using the md_run macro program, and the results were displayed by the md_analyze and md_analyzeres macro programs [41-43].

Results and discussion

Antitoxic activity of *S. caseolaris* leaves extract

Figure 1 demonstrates the effect of *S. caseolaris* leaves extracts (samples A - E) on TIG-1 fibroblast cell viability across concentrations ranging from 0 to 800 ppm. All extract treatments maintain cell viability above the commonly accepted cytotoxicity threshold of 70% at all tested concentrations, including the highest concentration, indicating low cytotoxicity toward normal human fibroblast cells. Among the tested samples, extract D consistently exhibited the highest cell viability across the concentration range, suggesting the lowest cytotoxic effect. In contrast, sample C showed a gradual decline in cell viability with increasing concentration, indicating relatively greater cytotoxicity compared with the other extracts, although still within the non-cytotoxic range. Samples A, B, and E demonstrated moderate and relatively stable viability values, suggesting minimal dose-dependent toxicity. These findings indicate that all tested *S. caseolaris* leaves extracts possess favorable biocompatibility toward TIG-1 cells under the evaluated conditions.

Figure 2 presents the probit regression analysis used to estimate LC₅₀ values of *S. caseolaris* leaves extracts (samples A - E) against *Artemia salina*. All extracts exhibited a concentration-dependent increase in probit mortality values, indicating that mortality rose proportionally with increasing extract concentration. However, none of the tested extracts reached probit values corresponding to 50% mortality within the tested concentration range, suggesting LC₅₀ values exceeding 10,000 ppm. Among the samples, extract D showed the highest probit values across concentrations, indicating relatively greater lethality compared with the other extracts, whereas extract C exhibited the lowest probit values, suggesting the least lethality. Nevertheless, all extracts remained within the non-toxic classification range according to standard brine shrimp lethality criteria. Collectively, the results of TIG-1 cell viability and *A. salina* lethality assays indicate that *S. caseolaris* leaves extracts exhibit low toxicity in both mammalian and non-mammalian biological models. All extracts maintained TIG-1 fibroblast viability above 70%, confirming non-cytotoxic behavior toward normal human cells, while LC₅₀ values exceeding 10,000 ppm in the brine shrimp assay further support their low general toxicity. Although minor differences in relative

toxicity were observed among extract types—with sample C showing comparatively lower fibroblast viability and sample D demonstrating slightly higher brine shrimp lethality—these variations did not reach toxic thresholds. The combined findings suggest that

aqueous and methanolic extracts of *S. caseolaris* leaves possess a favorable preliminary safety profile and may be suitable for further pharmacological investigation as potential natural anti-inflammatory agents.

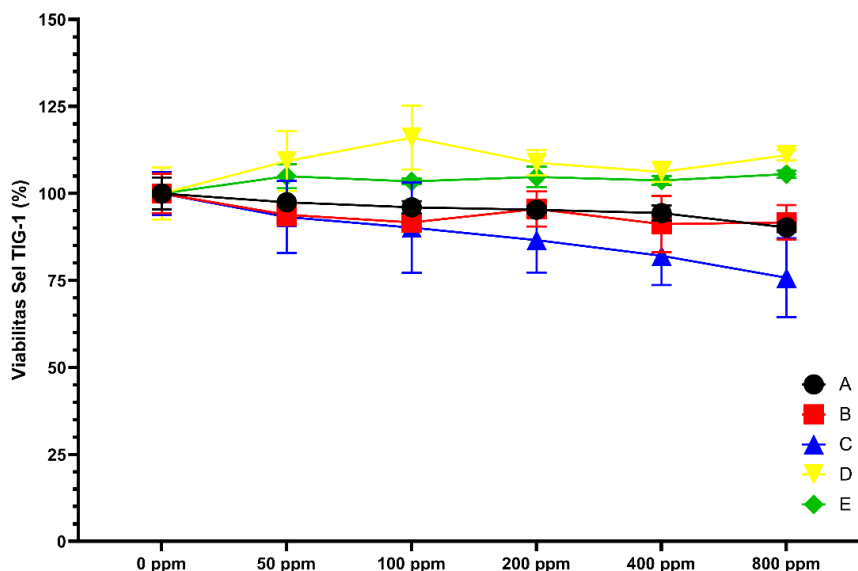


Figure 1 Percentage of growth inhibition on TIG-1 cell line by *S. caseolaris* leaves extract.

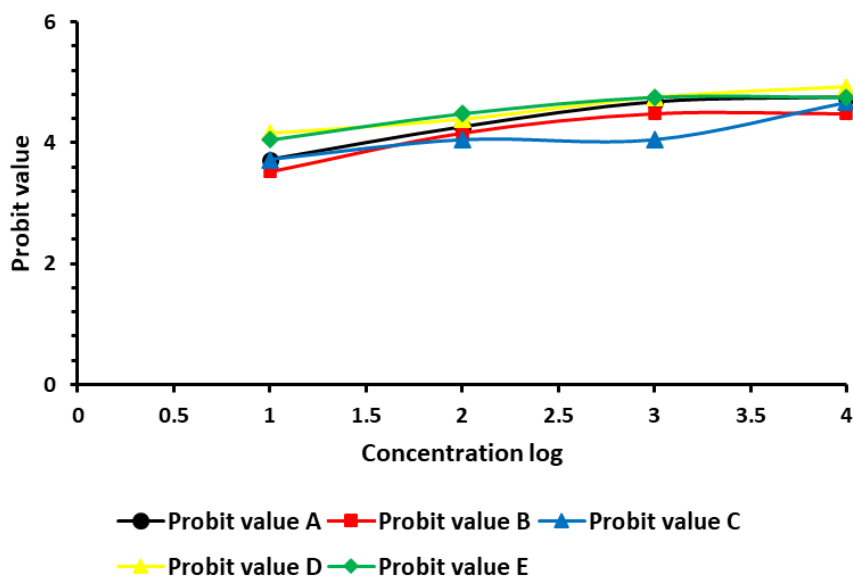


Figure 2 The probit value versus the concentration log for the determination of LC_{50} .

The combination of the assays provides an initial yet comprehensive evaluation of both mammalian cytotoxicity and general organismal toxicity [58]. The brine shrimp lethality assay showed LC_{50} values above 10,000 ppm for all tested extracts, classifying them as non-toxic [59]. Similarly, TEMPO-containing extracts

demonstrated over 70% cell viability in TIG-1 assays, indicating low cytotoxic potential. These findings are consistent with previous reports indicating low cytotoxicity of *Sonneratia* species, including *S. caseolaris* and *S. ovata*, toward normal cells under tested experimental conditions [60]. Taken together,

these results indicate that *S. caseolaris* extracts exhibited low preliminary toxicity, while TEMPO demonstrated favorable predicted pharmacokinetic characteristics and cytokine-binding behavior in computational analyses. Nonetheless, further pharmacological and toxicological studies are required to validate these findings and confirm the therapeutic potential and safety of TEMPO for future pharmaceutical development.

Drug-likeness and pharmacokinetic

The molecular weight, hydrogen bond acceptor, hydrogen bond donor, and LogP of the TEMPO were in

the appropriate amounts, which were 157.25 g/mol, 2, 1, and 1.84, respectively (**Table 1**). The LogP value of TEMPO falls within the range generally associated with favorable lipophilicity for membrane permeability (Optimal: $0 < \text{LogP} < 3$). TEMPO had a TPSA value of 23.47 Å (**Table 1**), which is consistent with predicted favorable intestinal absorption (TPSA value was more than 140). In addition, *in silico* prediction indicated that TEMPO has favorable aqueous solubility, indicated by a LogS value of 1.67 (**Table 1**). The absence of predicted CYP450 inhibition suggests a potentially low risk of CYP-mediated drug–drug interactions (**Table 2**).

Table 1 Drug-likeness of TEMPO.

Molecule	MW	H-Bond		Fraction Csp3	Rotatable Bonds	MR	TPSA	Consensus Log-P	ESOL Log-S	ESOL Class	Violation	
		Acceptors	Donors								Lipinski	Veber
TEMPO	157.25 g/mol	2	1	1	0	50.88	23.47	1.84	−1.67	Very Soluble	0	0

Table 2 Pharmacokinetic of TEMPO.

Molecule	Bioavailability score	GI Absorption	BBB permeant	Pgp substrate	Cyp inhibitor					Log - Kp (cm/s)	Alerts		Leadlikeness violation	Synthetic accessibility
					1A2	2C19	2C9	2D6	3A4		PAINS	BRENK		
TEMPO	0.55	High	Yes	No	No	No	No	No	No	−6.29	0	0	1	1.69

The potential of TEMPO in inhibiting pro-inflammatory agents

The results of molecular docking showed that the interaction of TEMPO with pro-inflammatory cytokines had a higher binding affinity value than the interaction of inhibitors-pro-inflammatory cytokines, namely −5.5, −4.4, and −4.3 kcal/mol, respectively. The interaction of inhibitors and TNF-α had the lowest affinity value (−9.1

kcal/mol) compared to the interaction of inhibitors and other pro-inflammatory cytokines, namely −5.8 kcal/mol and −6.4 kcal/mol (**Table 3**). These results suggest that TEMPO exhibits weaker predicted binding affinity toward the tested cytokines compared with reference inhibitors. The higher binding affinity value indicated that the bond between the ligand and receptor was weak [44].

Table 3 The interaction of TEMPO with TNF-α, IL6, and IL-1β pro-inflammatory cytokines.

Interaction			Binding affinity (kcal/mol)	Amino acid residues		
Ligand	CID	Receptor		Hydrogen interaction	Hydrogen bond distance (Å)	Hydrophobic interaction
TEMPO	549976	TNF-α	−5.5	-	-	Tyr59b, Ser60b, Gln61b, Tyr119a, Tyr119b, Leu120a, Leu120b, Gly121a, Gly121b, Gly122b, Tyr151b
		IL6	−4.4	Asn60	2.5	Ala56, Ala58, Asn61, Leu62, Leu64, Lys66, Leu165, Arg168, Ser169,
		IL-1β	−4.3	Lys63 Asn66	3.3 2.3	Ser43, Leu62, Glu64, Lys65, Leu67, Tyr68, Pro87, Tyr90, Pro91

Interaction			Binding affinity (kcal/mol)	Amino acid residues		
Ligand	CID	Receptor		Hydrogen interaction	Hydrogen bond distance (Å)	Hydrophobic interaction
Inhibitor	5327044	TNF- α	-9.1	-	-	Leu55d, Leu57a, Leu57b, Ile58, Tyr59a, Tyr59b, Ser60a, Ser60b, Gln61a, Gln61b, Tyr119a, Tyr119b, Leu120a, Leu120b, Gly121a, Gly121b, Gly122a, Gly122b, Tyr151a, Tyr151b, Ile155, Leu157d
	447043	IL6	-5.8	Gln28 Ser118	3.2, 3.3 3.1	Leu19, Glu23, Arg24, Lys27, Tyr31, Ile32, Ala114, Met117, Val121, Phe125
	139595263	IL-1 β	-6.4	Ser5 Tyr68	2.3, 2.4 2.4	Ala1, pro2, Val3, Arg4, Leu6, Asn7, Ser43, Ser45, Leu62, Lys63, Glu64, Asn66, Leu67, Asp86, Pro87, Lys88, Tyr90, Pro91,

Visualization with Discovery Studio aims to determine the binding site of the interaction of TEMPO and inhibitors on pro-inflammatory cytokines. TEMPO had the same binding site as inhibitors on pro-inflammatory cytokines, TNF- α and IL-1 β (**Figures 3 and 5**). Ser43, Leu62, Lys63, Glu64, Lys65, Asn66, Leu67, Tyr68, Pro87, Tyr90, Pro91 were the same 11 amino acids involved in the TEMPO-IL-1 β and inhibitor-IL-1 β interactions. Lys63 and Asn66, were two amino acid residues that interact through hydrogen bonds in the TEMPO-IL-1 β interaction and hydrophobic bonds in the inhibitor-IL-1 β interaction (**Table 3**). Not only that, Tyr68 was another amino acid that interacted through hydrophobic bonds in the

TEMPO-IL-1 β interaction and interacted through hydrogen bonds in the inhibitor-IL-1 β interaction (**Table 3**). Other results showed that TEMPO had a different binding site with the inhibitor on the pro-inflammatory cytokine IL6 (**Figure 4**). TEMPO may bind IL-6 through a different mode than the reference inhibitor, while showing overlapping predicted binding regions with reference ligands for TNF- α and IL-1 β . However, as molecular docking only predictive structural insights, no definitive inhibition mechanism can be established without biochemical validation. Nevertheless, TEMPO demonstrated predicted interactions with IL-6 through hydrogen and hydrophobic interactions [45].

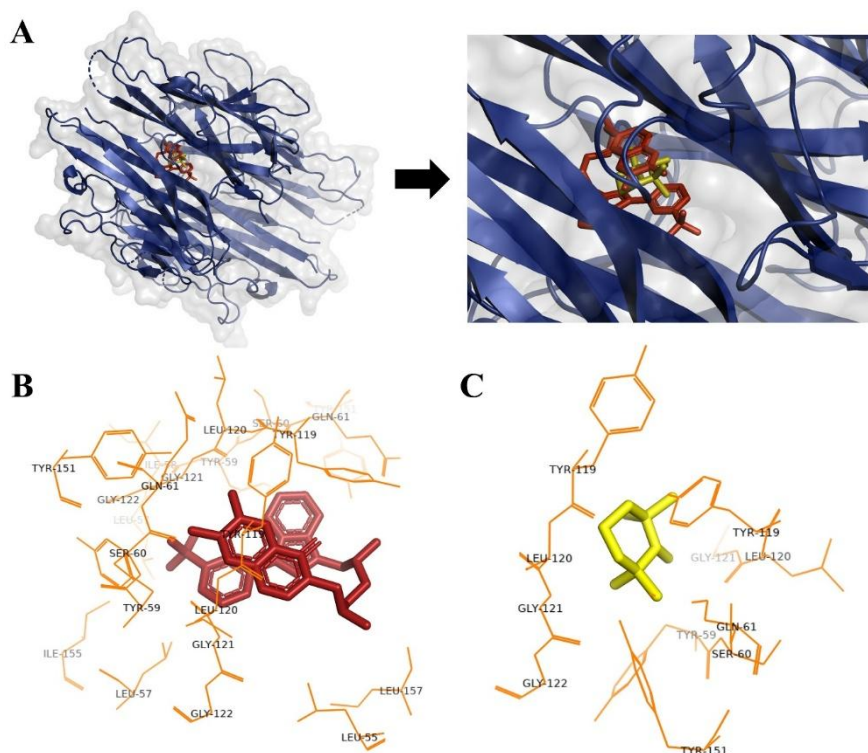


Figure 3 Interaction of ligands-TNF- α and the amino acid residues involved. (A) Molecular docking visualization between inhibitor and TEMPO with TNF- α . (B - C). The amino acid residues involved in the interaction inhibitor-TNF- α and TEMPO-TNF- α . TNF- α was presented as a blue ribbon. Inhibitor (red) and TEMPO (yellow) were presented as sticks).

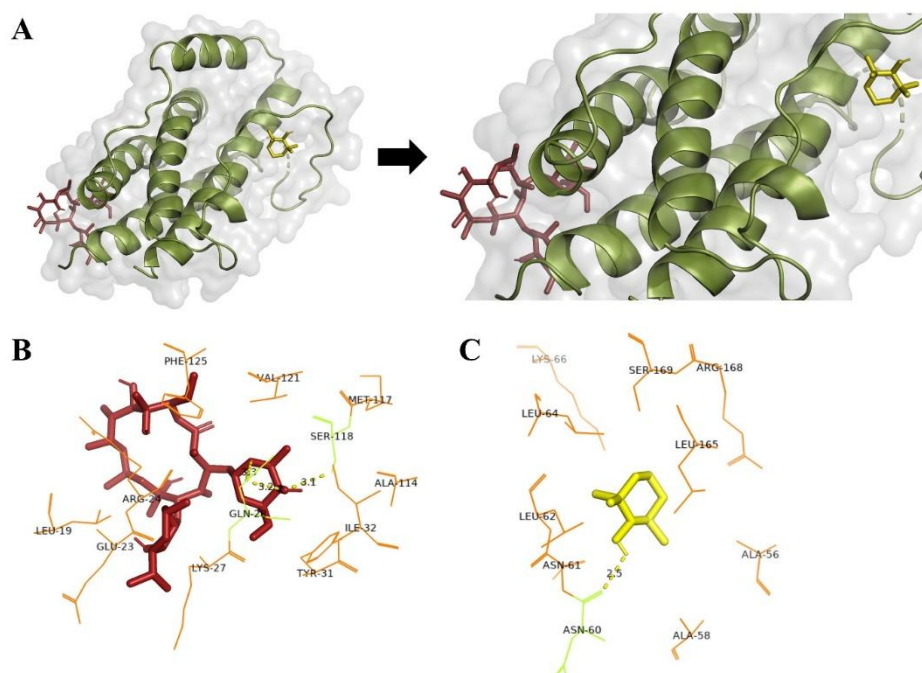


Figure 4 Interaction of ligands-IL6 and the amino acid residues involved. (A) Molecular docking visualization between inhibitor and TEMPO with IL6. (B - C). The amino acid residues involved in the interaction inhibitor-IL6 and TEMPO-IL6. IL6 was presented as a green ribbon. Inhibitor (red) and TEMPO (yellow) were presented as sticks).

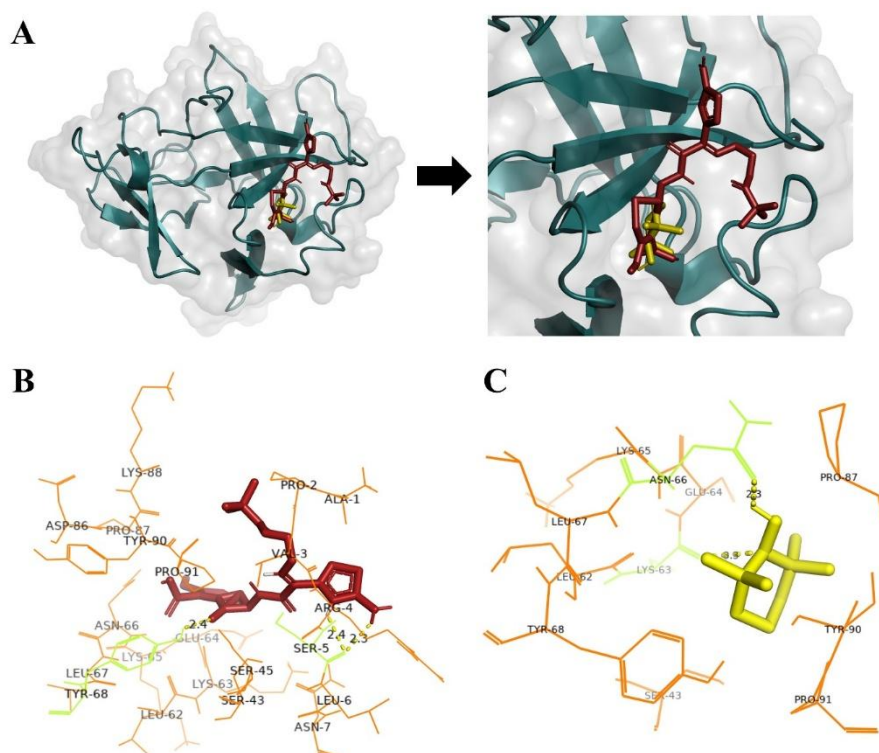


Figure 5 Interaction of ligands- IL-1 β and the amino acid residues involved. (A) Molecular docking visualization between inhibitor and TEMPO with IL-1 β . (B - C). The amino acid residues involved in the interaction inhibitor-IL-1 β and TEMPO-IL-1 β . IL-1 β was presented as a cyan ribbon. Inhibitor (red) and TEMPO (yellow) were presented as sticks).

Molecular dynamic simulation

The results of the molecular dynamic simulation can be seen in **Figures 6 - 8**. The pro-inflammatory cytokine IL-1 β complexed with TEMPO had the most stable RMSD value compared to the TNF- α -TEMPO and IL6-TEMPO complexes, which was less than 3 Å and the RMSD value gets smaller starting at 25 ns until the end of the simulation. Meanwhile, the RMSD value of IL-1 β complexed with inhibitor ligand showed a different trend, since the beginning of the simulation until the time of 25 ns experienced fluctuations but after

that the RMSD value was stable below 3 Å until the end of the simulation (**Figure 6(C)**). These results suggest that the IL-1 β -TEMPO complex exhibited greater structural stability during the simulation than the IL-1 β -inhibitor complex. A similar trend was observed for TNF- α , where the TEMPO-bound complex showed comparatively lower RMSD fluctuations than the inhibitor-bound complex (**Figure 6(A)**). The RMSD value of not more than 3 Å indicated a stable protein during the simulation and if it was more than 3 Å then the protein had undergone some structural changes [46].

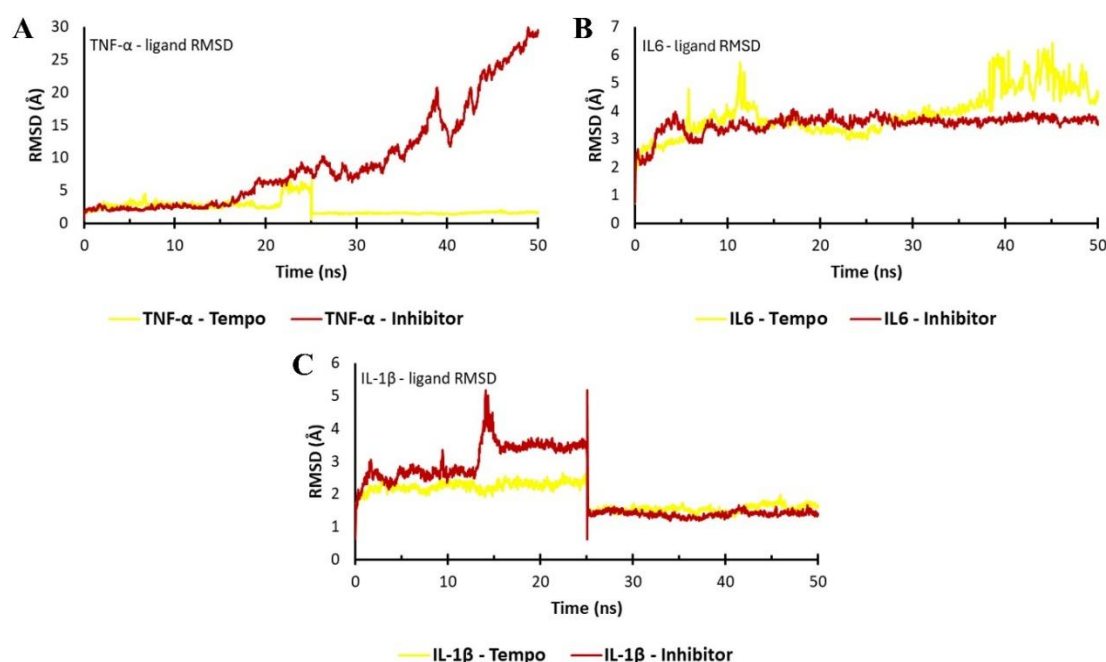


Figure 6 RMSD values indicated the stability of protein-ligand complex interactions. (A) The RMSD values of the TNF- α -TEMPO and TNF- α -inhibitor. (B) The RMSD values of the IL6-TEMPO and IL6-inhibitor. (C) The RMSD values of the IL-1 β -TEMPO and IL-1 β -inhibitor. TNF- α indicated Tumor Necrosis Factor Alpha; IL6, Interleukin-6; IL-1 β , Interleukin-1-Beta.

TNF- α complexed with inhibitors showed unstable amino acid residue conformations compared to when complexed with the TEMPO compound (**Figure 7(A)**). These results caused the RMSD value of TNF- α -inhibitor to tend to be higher and unstable at times above 15 ns until the end of the simulation compared to the RMSD value of TNF- α -TEMPO (**Figure 6**). IL6 complexed with TEMPO, there were unstable amino acids, namely Arg30, Lys54, Leu57, Asn60, and Lys131 (**Figure 6(B)**). These residues caused the RMSD value of the IL6-TEMPO complex to be more unstable than the IL6-inhibitor complex (**Figure 6(B)**). In IL-1 β

complexes with inhibitors, there were unstable amino acid residues, such as Ala1, Glu50, Asn53, Lys88, Gly139, and Ser153 (**Figure 7(C)**). This caused the RMSD value of the IL-1 β -inhibitor complex to tend to be unstable compared to the IL-1 β -TEMPO complex (**Figure 6(C)**). A high RMSF value indicated that the amino acid was unstable or had high fluctuations during the simulation [47]. RMSD and RMSF analyses provide complementary information regarding conformational stability and flexibility of protein-ligand complexes during simulation.

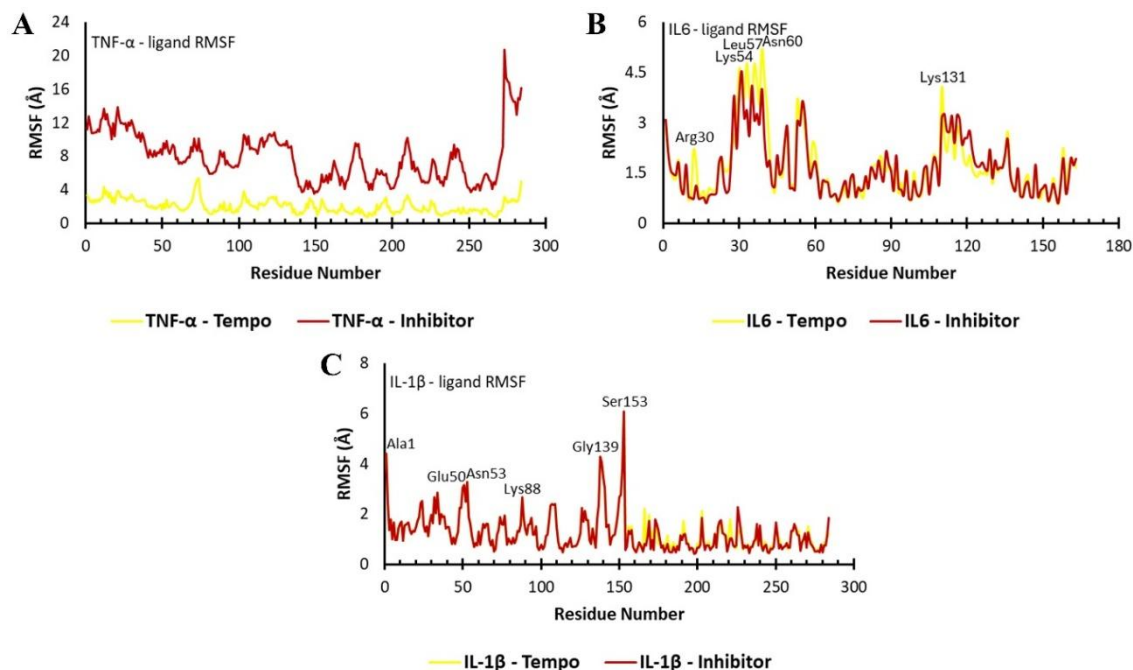


Figure 7 RMSF value indicated the stability of each amino acid residue during the simulation. (A) The TNF- α -inhibitor complex had residues with higher flexibility than the TNF- α -TEMPO complex. (B) Asn50 and Leu57 residues of the IL6-TEMPO complex had high flexibility. (C) Ser153 residue of the IL-1 β -inhibitor complex had high flexibility. High flexibility indicated instability of residue. TNF- α indicates Tumor Necrosis Factor Alpha; IL6, Interleukin-6; IL-1 β , Interleukin-1-Beta.

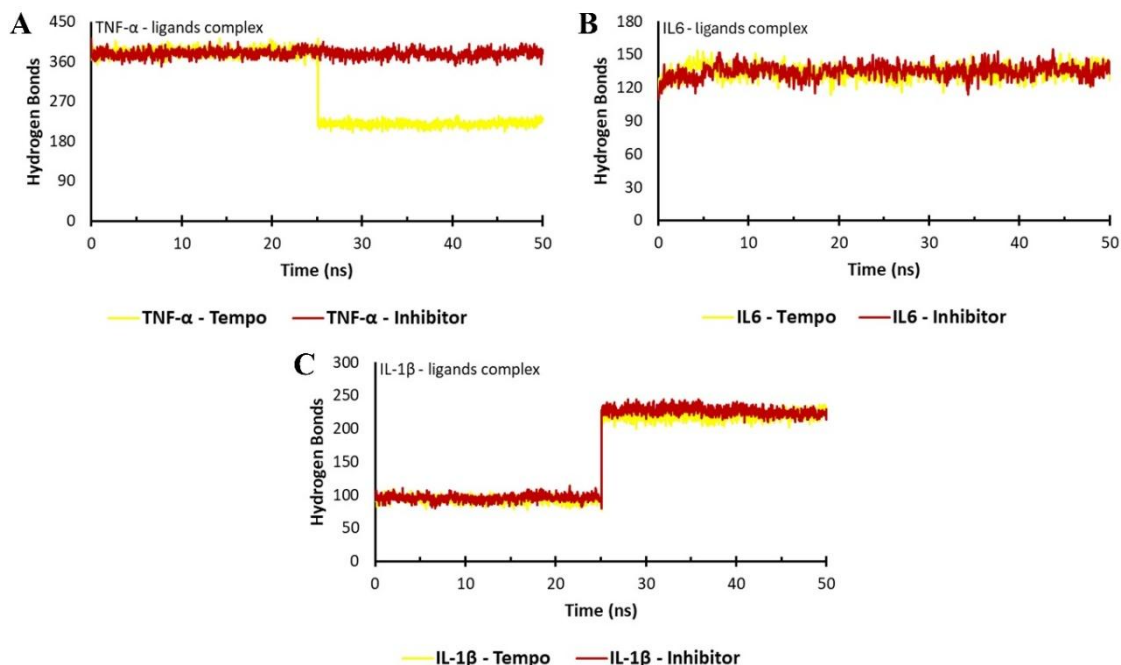


Figure 8 The number of hydrogen bonds during the simulation indicated the stability of the complex structure. The number of hydrogen bonds in TNF- α -ligands (A), IL6-ligands (B), and IL-1 β -ligands (C). These results indicated the structural stability of the pro-inflammatory cytokines-ligands complexes. TNF- α indicates Tumor Necrosis Factor Alpha; IL6, Interleukin-6; IL-1 β , Interleukin-1-Beta.

Drug-likeness evaluation provides an initial qualitative framework for assessing the potential of a molecule to act as an orally active drug, primarily based on its bioavailability and physicochemical properties [37]. These characteristics strongly influence absorption, distribution, metabolism, and excretion (ADME) processes, which are crucial for the pharmacokinetic behavior of a candidate compound. In this study, TEMPO, a compound identified in *S. caseolaris* leaf extract through LC-HRMS [30], was further analyzed for its ADME properties using *in silico* methods. The prediction results indicated that TEMPO fulfills Lipinski's "rule of five" [48], suggesting favorable oral bioavailability. Its high aqueous solubility supports this potential, as solubility enhances drug absorption and facilitates formulation [49,50]. Although TEMPO exhibited low skin permeability with negative log K_p values, this parameter has limited relevance to oral drug development. Furthermore, the predicted interactions of TEMPO with cytochrome P450 isoenzymes highlight its possible involvement in metabolic biotransformation, a key process in drug elimination [51].

Building upon its favorable drug-likeness, *in silico* molecular docking and dynamics simulations were performed to investigate the anti-inflammatory potential of TEMPO. Inflammation is a vital defense mechanism involving cytokines such as TNF- α , IL-6, and IL-1 β ; however, chronic overproduction of these mediators leads to pathological inflammatory responses and tissue damage [52]. Conventional anti-inflammatory drugs can effectively suppress these responses, yet prolonged use often results in adverse effects [53,54]. Therefore, identifying natural compounds with safer anti-inflammatory properties remains an important pharmacological objective.

The docking results revealed that TEMPO occupied overlapping binding regions with reference inhibitors in TNF- α and IL-1 β , suggesting possible interaction with functionally relevant sites. The presence of shared amino acid residues—such as Tyr59b, Ser60b, and Tyr119b—in the TEMPO–TNF- α complex indicates comparable ligand–receptor interactions [39]. Although TEMPO binds IL-6 outside the active site, the interaction could still alter cytokine conformation, potentially reducing its biological activity. Both hydrophobic and hydrogen bond

interactions were found to play essential roles in stabilizing these ligands–protein complexes, consistent with previous findings that these bonds enhance binding affinity and structural stability [55].

Molecular dynamics simulations further confirmed the stability of the TEMPO–cytokine complexes. The RMSD, RMSF, and hydrogen bond analyses demonstrated that the TNF- α –TEMPO and IL-1 β –TEMPO complexes maintained structural stability throughout the simulation period, with RMSD values below 3 Å, indicating limited conformational fluctuation. Interestingly, although the TNF- α –TEMPO complex exhibited fewer hydrogen bonds than the inhibitor complex, it remained more stable overall. This suggests that the stability was not solely determined by the quantity but also by the quality and geometry of hydrogen bonding within the binding pocket [56,57]. These observations suggest that TEMPO may interact with TNF- α and IL-1 β at inhibitor-overlapping regions, while its interaction with IL-6 may involve a distinct binding site. Experimental studies are required to determine the actual mechanism of action.

Conclusions

The aqueous extracts of *S. caseolaris* leaves exhibited low toxicity in preliminary TIG-1 cell viability and *Artemia salina* lethality assays. TEMPO, tentatively identified in the water extract of *S. caseolaris* leaves, demonstrated favorable predicted interactions with pro-inflammatory cytokine targets in molecular docking and molecular dynamics simulations. These findings suggest that *S. caseolaris* leaves extract may contain compounds with anti-inflammatory potential; however, further compound isolation, structural confirmation, and experimental pharmacological validation are required to confirm the bioactivity and therapeutic relevance of TEMPO.

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The authors acknowledge the use of generative AI tools (e.g., Grammarly) in the preparation of this manuscript, specifically for language editing and grammar correction. No content generation or data interpretation was performed by AI. The authors take full responsibility for the content and conclusions of this work.

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