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Anti-Inflammatory Activity of Quts Al-Hindi Extract (*Saussurea Lappa*) Using Erythrocyte Membrane Stability and Prediction of Its Selectivity for COX-2 and INOS Enzymes

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Abstract: An immune system reaction known as inflammation can damage tissue by rupturing mast cells, followed by released inflammatory mediators such as nitric oxide (NO) and increased prostaglandin secretion by cyclooxygenase proteins. One of the prospective plants that contain several bioactive components applied in medication development, such as anti-inflammatory agents, is Quts al Hindi (*Saussurea lappa*). At the moment, the use of natural products as anti-inflammatory agents is being developed. With the help of the erythrocyte membrane stability method and in silico analysis, this study aims to determine the anti-inflammatory effectiveness of the root extract of *Saussurea lappa*. The methods of determining the anti-inflammatory activity involved determining the stability of the erythrocyte membrane by determining the drug's ability to prevent red blood cell lysis caused by the induction of a hypotonic solution contrasted with the positive control of diclofenac sodium. The Poisson–Boltzmann molecular mechanics calculation technique and surface area (MM-PBSA) were used to simulate docking and molecular dynamics. The results showed that the activities of extracts at doses of 125, 100, 75, 50, and 25 ppm were 91.55%, 90.64%, 89.44%, 87.98%, and 84.85%, respectively, compared with diclofenac sodium as a positive control at the same concentration, which was 85.67%, 84.60%, 83.02%, 82.30%, and 81.71%, respectively. The results of molecular docking and molecular dynamic simulations of ten compounds of *Saussurea lappa* showed that cosmic acid and costunolide are more selective into the active site of COX-2 than the iNOS enzyme, with free energy (ΔG) values were -9.6 and -9.4 and 7.2 and 7.1 kcal/mol, respectively. The novelty of this study is the anti-inflammatory activity of *Saussurea lappa* compounds obtained by interaction mechanisms and molecular dynamics against COX-2 and iNOS using the erythrocyte membrane stability method.

Keywords: anti-inflammatory, Quts Al Hindi, membrane stabilization.

利用紅血球膜穩定性研究印地語萃取物（雪蓮）的抗發炎活性並預測其對環加氧酶-2和誘導型一氧化氮合成酶酶的選擇性

摘要：一種稱為發炎的免疫系統反應會破壞肥大細胞，然後釋放一氧化氮等發炎介質，

並透過環氧合酶蛋白增加前列腺素分泌，從而損害組織。印地語(雪蓮)是含有多種用於藥物開發的生物活性成分（例如抗發炎劑）的潛在植物之一。目前，正在開發使用天然產物作為抗發炎劑。借助紅血球膜穩定性方法和電腦分析，本研究旨在確定雪蓮根萃取物的抗發炎功效。測定抗發炎活性的方法包括透過測定藥物防止低滲透壓溶液誘導引起的紅血球溶解的能力來確定紅血球膜的穩定性，與雙氯芬酸鈉的陽性對照相比。使用泊松玻爾茲曼分子力學計算技術和表面積來模擬對接和分子動力學。結果表明，與雙氯芬酸鈉陽性相比，125、100、75、50、25百萬分之一萃取物活性分別為91.55%、90.64%、89.44%、87.98%、84.85%相同濃度下對照，分別為85.67%、87.98%、84.85%相同濃度下對照，分別為85.67%、87.60%、83.02%、82.30%和81.71%。對雪蓮10種化合物的分子對接和分子動力學模擬結果表明，宇宙酸和木香烯內酯對環加氧酶2活性位點的選擇性高於誘導型一氧化氮合成酶酶，自由能值分別為9.6、9.4和7.2，分別為7.1千卡/摩爾。本研究的新穎之處在於使用紅血球膜穩定性方法透過相互作用機制和分子動力學對環加氧酶2和誘導型一氧化氮合成酶獲得了雪蓮化合物的抗發炎活性。

关键词：抗發炎，印地語，膜穩定。

1. Introduction

Wounds and infections cause inflammatory processes in the tissue; therefore, immune cells and cytokines release nitric oxide (NO), prostaglandin (PG), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α) [1, 2]. Prolonged inflammation can develop into chronic inflammation, which is a cause of cancer. As well documented, the inflammation process involves the sequential activation of signaling molecules, proinflammatory mediators, such as prostaglandins (PGs) and nitric oxide (NO), which are generated by cyclooxygenases (COXs) and inducible nitric oxide synthase (iNOS), respectively [3]. Inflamed areas can be exposed to free radicals such as hydroxyl (OH), superoxide (O_2), and peroxyl (OOH, OOR) and cause excessive damage. Furthermore, the inducible transcription factor (NF-kB) regulates the expression of several genes, such as iNOS, COX-2, and TNF-a, which are involved in inflammatory responses at the transcriptional level. Here, iNOS is responsible for the overproduction of endogenous free radical nitric oxide (NO) in the inflammation site [4]. Cyclooxygenases-2 (COX-2) is a stimulus enzyme in inflammation; therefore, the activity inhibition of this enzyme will be a target for inflammation treatment [2, 3]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to be able to treat inflammatory diseases. This drug is successful as an anti-inflammatory agent but has failed to inhibit COX-2 [3, 6]. Furthermore, long-term consumption of NSAIDs is known to cause stomach irritation, hemorrhage, kidney, bronchus, cardiovascular system, and perforation [6, 7]. Natural products with anti-inflammatory activity apply as traditional medicines for inflammatory conditions such as fever, pain, migraines, and arthritis. As the inflammatory basis of the disease became obvious, anti-inflammatory foods and food products (traditional medicines) became a more prevalent interest. In general, the treatment used to treat inflammation is NSAID-class drugs such as diclofenac sodium. These drugs are called modern drugs and can carry the risk of toxicity for prolonged use. Therefore, people use traditional medicines for therapeutic needs, which have many benefits and relatively low side effects. Several plants contain bioactive compounds that have medicinal properties, one of which is Quts al Hindi.

Quts al Hindi (*Saussurea lappa*), or Indian wood, is empirically capable of curing seven diseases, one of which is related to the lungs. It also has anti-inflammatory properties that prevent inflammation. One of the pathogenic compounds is the product of microorganisms, especially endotoxins or lipopolysaccharide (LPS) components released by gram-negative bacteria that can trigger inflammatory reactions, stimulating macrophage cells to produce cytokines in inflammatory tissues [8–10].

Based on previous research, the methanolic extract of Quts al Hindi (*S. lappa*) has been assessed for its anti-inflammatory activity. It exhibits an inhibitory (50%) effect on neutrophil chemotactic factors induced by cytokines. Anti-inflammatory activity was also demonstrated by sesquiterpene lactones in the *S. lappa* fraction at a concentration level of 0.05–0.2 g/kg [10]. Other studies also found that *S. Lappa* contains several marker compounds in the form of allantolactone, caryophyllene, costalis acid, costunolide,

dehydrocostuslactone, and cyclaropicrin. In vitro, the anti-inflammatory effects of cynaropicrin and a sesquiterpene lactone from *Saussurea lappa* impact the release of tumor necrosis factor (TNF-α) and nitric oxide (NO) and lymphocyte proliferation. Cynaropicrin in the inflammatory response inhibits tumor necrosis factor-alpha (TNF-) and NO and CD4+ and CD8+ proliferating lymphocytes by conjugation with sulfhydryl groups on target proteins [10–14].

The basic principle of the in silico method approach is to bind the ligand or drug compound to a target in the form of macromolecular affinity, where the smaller the free, the better the activity between the ligand receptors [15, 16]. The analysis results from this study can validate the results obtained or as a first step to examine a system or problem that is still very difficult to perform with conventional methods. Molecular docking was employed to analyze the interaction of compounds and ligands, which is a computational procedure that attempts to efficiently predict the non-covalent bond between macromolecules (targets) and small molecules (ligands). The purpose of this method is to predict the conformation of the bonds that occur and the affinity of the bonds formed. This prediction is essential because it is a virtual screening tool for compounds with the potential to be new drugs [17, 18].

Various methods can test the anti-inflammatory activity of a drug, its chemical constituents, and herbal preparations to determine its anti-inflammatory activity or potential. These techniques include uncoupling oxidative phosphorylation (ATP biogenesis associated with respiration), inhibition of protein denaturation, stabilization of erythrocyte membranes, stabilization of lysosomal membranes, fibrinolytic assays, and platelet aggregation [19]. The membrane of human erythrocytes or erythrocytes is analogous to the lysosomal membrane, and its stabilization assumes that the extract can also stabilize the lysosomal membrane. Lysosomal membrane stabilization is essential in limiting the inflammatory response by inhibiting the release of lysosomal constituents of active neutrophils, such as bactericidal enzymes and proteases, leading to inflammation and further tissue damage upon extracellular release [20, 21].

We were interested in conducting this research because there are no publications regarding samples using the in vitro method to determine the stability of the erythrocyte membrane. Several tests of the activity of compounds from samples as anti-inflammatory agents have been reported, but not their selectivity for the COX-2 receptor.

2. Materials and Methods

The materials used in this study were (In Vitro) reagents were the roots of the plant QUTS al Hindi, blood, EDTA, 96% ethanol, diclofenac sodium, aqua distilled, $Na_2HPO_4.2H_2O$, $NaH_2PO_4.H_2O$, NaCl, chloroform, acetic anhydrous acid, concentrated sulfuric acid, HCl, and iron (III) chloride. (In Silico) target protein or receptor, namely COX-2 (PDB ID 3NT1) with a resolution of 1.73, which binds to the ligand (2S)-2-(6-methoxynaphthalen-2-yl) propanoic acid) or is called naproxen, whereas iNOS (PDB: 3E7G) with a resolution of 2.20 binds to the ligand (Ethyl 4-[(4-methylpyriclin-2-Yl)Amino]pyredirine-1-carboxylate). The crystal structure chosen must have the best or lower resolution value and be R-free and R-value lower than 0.25. The test ligand is a chemical compound found in Quts al Hindi (*Sausserea lappa*) with a 3D structure obtained from the PubChem database. Then, it is optimized using ChemDraw and 3D chem.

2.1. Test Using Erythrocyte Membrane Stability as an Anti-Inflammatory (In Vitro Methods)

Stage I: 50 g of simplicia of *Saussurea lappa* are put into a maceration container, adding ethanol until simplicia is soaked and set aside for 3×24 h by stirring several times safe from sunlight. After 3×24 h, the simplicia is strained, and the dreg is re-soaked with a new solution [23] until the extraction process is perfect using filter paper concentrated until a condensed extract is obtained [24].

Stage II: Preparation of a suspension (10% v/v) of erythrocytes was as follows. The blood sample was placed into a centrifugation tube containing Alsever's solution in the same ratio and then centrifuged at 3,000 rpm for 15 min at room temperature. The supernatant formed was carefully separated from the RBCs using a sterile pipette. The precipitated blood cells were washed with an isovaline solution and centrifuged again. The washing and centrifugation processes were repeated five times until the supernatant was clear. The red blood cell volume was measured and suspended with an isovaline solution to obtain a red blood cell suspension concentration of 10% v/v.

Stage III: An in-vitro anti-inflammatory activity test was performed using the red blood cell membrane stabilization method and compared with a standard solution (diclofenac sodium 100 µg/mL). The test mixture consisted of 2 mL hypotonic saline, 1.0 mL 0.15 M sodium phosphate buffer (pH 7.4), 0.5 mL (10% v/v) red blood cell suspension, and 1.0 mL test sample and standard solution. The test mixture was incubated at 370℃ for 30 min, and then the solution was centrifuged at 3000 rpm for 30 min. Measurement of the absorbance of the solution was at a wavelength of 560 nm. The percentage of hemolysis inhibition calculation used the following formula:

$$\% \text{ inhibition} = \frac{A1-A2}{A1} \times 100\ \%$$

where *A1* - absorbance of the test control solution, *A2* - absorbance of the test solution/test standard solution.

2.2. In Silico Methods

2.2.1. Molecular Docking Using Autodock

Docking simulation of COX-2 (3NT1) and iNOS (3E7G) receptors and test ligands is performed by opening the command prompt and then entering the docking command according to Autodock Vina. The results obtained used a notepad to determine the bond energy (ΔG) and the lowest inhibition constant (Ki) between natural ligands. The docking process starts, and wait until the running process is complete. The docking results will appear after the running process is complete, and the binding affinity (ΔG) (kcal/mol) value will appear. To save the docking complex on the column navigator, right-click and save in the PDB format (save as PDB) [29-31].

Validation is performed by re-docking or re-tethering natural ligands previously removed from the target receptor. The re-docking process used a grid box for COX-2 x = -40.699; y = -51.5; z = -22.4, grid side = 40×40×40 and iNOS x = 55.392526; y = 21.342268; z = 79.163737, grid site = 12×16×36 with space 0.375 Å accomplished by opening a command prompt and entering the docking command according to Autodock.

Analysis of the docking results to determine the interaction between the receptor and the ligand used Discovery Studio software. It was carried out by entering the docking complex in the .pdb file and observing the interactions of amino acid residues, hydrogen bonds, bond distances, and hydrophobic interactions that occurred.

Chemical compounds from *S. lappa* by looking at the ΔG value because of docking through the in silico screening method using Autodock software and visualization using Discovery Studio. Where the binding affinity or Gibbs free energy (ΔG) generated in docking calculates the binding energy between the ligand and the receptor, the lower the value of ΔG describes the better the affinity between the ligand and the receptor [22].

2.2.2. Molecular Dynamics Simulation

The molecular dynamics simulation used Gromacs 2016.3 software with AMBER99SB-ILDN as a force field in its simulations and the AnteChamber Python Parser interface (ACPYPE) being used to parameterize the topologies, atomic types, and charges [36]. The particle mesh Ewald method determined the electrostatic force over distance [24]. Na^+ and Cl^- ions were added to the system as a simulation setup neutralizer. Solvation used the TIP3P water cube model [37]. The simulation preparation stage included the minimization step, heating to 310°K, and temperature and pressure equilibrations, followed by the simulation process. Furthermore, the molecular dynamics simulation runs were performed for 100 ns and were updated every 2 fs. Finally, the molecular dynamics simulation trajectories were analyzed by obtaining their RMSD, root-mean-square fluctuation (RMSF), and molecular mechanics Poisson–Boltzmann and surface area (MM-PBSA) binding free energy calculations.

2.2.3. MM-PBSA Binding Free Energy Calculations

Free energy calculations used the MM-PBSA method that was available in the GROMACS software package and prepared using the g_mmpbsa tool [37]. The MM-PBSA was applied to predict the average binding free energies and standard deviations of all energetic components, including the binding energy, the polar solvation energy (ΔG_{polar}), the solvent-accessible surface area (SASA; $\Delta G_{nonpolar}$), the electrostatic interaction (ΔE_{elec}), and the Van der Waals interaction (E_{vdW}). On the other hand, the *full_energy.dat* function contained the values of the energetic terms as a function of time, and the latter were plotted using the grace software. Furthermore, visualization of the energy contribution of each residue to its structure used the VMD program.

Fig. 1 shows the flow chart of this research methodology.

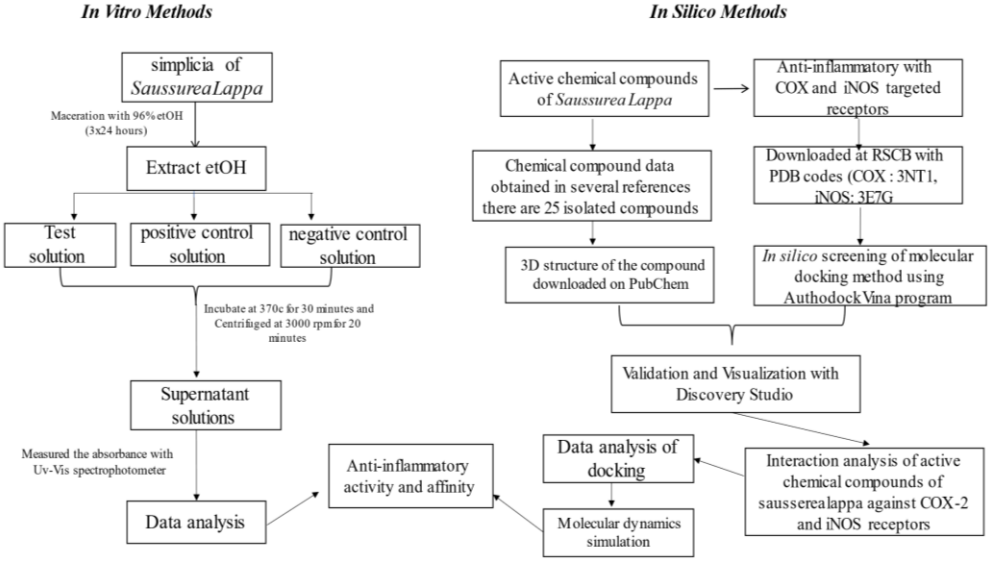


Fig. 1 Flowchart of the study

3. Results and Discussion

3.1. Results of Anti-inflammatory Activity Using the Eritrosit Membrane Stability Test of *Saussurea Lappa* (In Vitro)

The stabilizing method of red blood cell membrane is used because the cell structure is similar to that of lysosomes, where erythrocytes have a membrane that wraps hemoglobin. When the membrane is broken, the hemoglobin inside will come out, and hypotonic stress will occur, which disrupts the stability of the membrane. The amount of hemolysis that occurs in the red blood cell membrane induced by a hypotonic solution can determine the anti-inflammatory activity of extract from the roots of the QUTS al Hindi (*Saussurea lappa*) plant.

The test solution is the extract results from *Saussurea lappa* prepared at several concentrations. Table 1 shows the anti-inflammatory activity of the ethanol extract from the decrease in absorbance, where the lower the absorbance value, the less hemoglobin is released so that the more stable membrane and the anti-inflammatory activity is greater. A large absorbance value results in a large percent stability value.

Table 1 Value of absorbance and percentage of erythrocyte membrane stability from abstract and diclofenac sodium

Sample	Concentration (ppm)	Ln C	Absorbance			±SD, n=3	% of inhibition	IC_{50} µg/mL
			1	2	3			
Ethanol extract	125	4.83	0.08	0.071	0.075	0.075	91.55	1.762
	100	4.60	0.078	0.08	0.078	0.079	90.63	
	75	4.32	0.097	0.082	0.087	0.089	89.44	
	50	3.91	0.111	0.081	0.111	0.101	87.98	
	25	3.22	0.125	0.132	0.125	0.127	84.84	
Diclofenac sodium	125	4.828	0.189	0.081	0.091	0.120	85.675	1.861
	100	4.605	0.198	0.095	0.095	0.129	84.603	
	75	4.317	0.142	0.143	0.143	0.143	83.016	
	50	3.912	0.152	0.142	0.152	0.149	82.302	
	25	3.219	0.152	0.151	0.158	0.154	81.706	

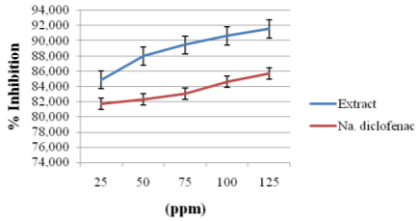


Fig. 2 Histogram of inhibition with different concentrations of erythrocyte membrane stability

Based on Table 1 and Fig. 1, the concentration of 125 ppm showed the highest percentage inhibition of the extract, even greater than that of the positive control. These data indicate that the stability of the red blood cell membrane increases with the concentration of the extract. The inhibitory ability of the *Saussurea lappa* extract is due to the presence of a chemical compound that can stabilize red blood cell membranes, but no data have been reported that certain saponins and flavonoids have a stabilizing effect on lysosomal membranes both in vivo and in vitro. Therefore, this research can be used as a reference to see the potential anti-inflammatory properties of QUTS al Hindi plant roots.

3.2. Molecular Docking Results of *S. Lappa* Compounds against COX-2 and iNOS Receptors (In Silico)

3.2.1. Molecular Docking Simulation Test Results

Molecular docking is an in silico method to analyze the interaction between receptors and ligands. The purpose of this method is to predict the conformation of the bond that occurs and the affinity of the formed bond. This prediction is essential because it is a virtual screening tool for compounds that can be new drugs. In silico prediction is a method to predict and confirm drug design. This method has advantages such as being inexpensive, time-consuming, and minimally isolating inactive compounds [5, 30]. The research used Autodock software based on MGL Tools between selected compounds from *S. lappa*.

The parameter used is the root mean square deviation (RMSD), which is a parameter that describes how much the protein–ligand interaction changes in the crystal structure before and after docking. The method can be said to be valid if the RMSD value is ≤ 2 Å. Based on the results of the validation process, the overlay form of naproxen as a COX-2 inhibitor (PDB: 3NT1) has similarities, as indicated by an RMSD value of 1.008 Å (Fig. 2). Fig. 3 shows the validation results of the iNOS substrate (PDB: 3E7G) with the native ligand ethyl 4-[(4-methylpyriclin-2-Yl)amino]pyredirine-1-carboxylate to obtain an RMSD value of 0.723 Å.

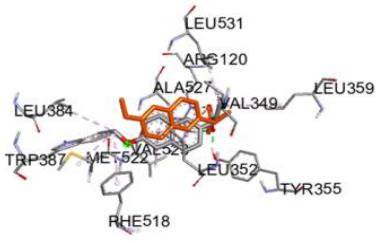


Fig. 3 Naproxen interaction results (shown in orange) and the naproxen re-docking results (shown in gray) in the COX-2 receptor-binding pocket (3NT1)

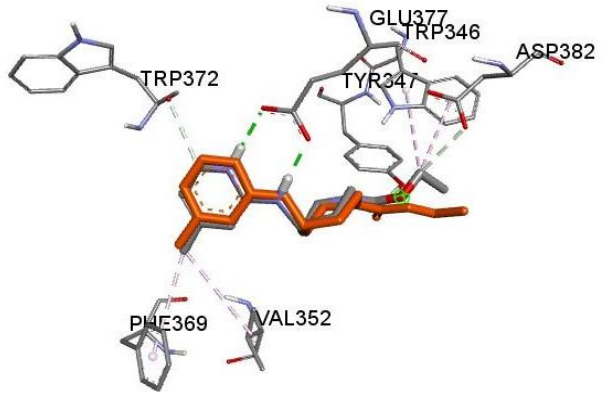


Fig. 4 The CID11149707 code interaction results (shown in orange) and the CID11149707 re-docking results (shown in gray) in the iNOS receptor-binding pocket (3E7G)

The in silico approach commonly applies to the prediction and confirmation of drug design. This method has advantages such as being inexpensive, time-consuming, and minimally isolating inactive compounds [5, 28]. The study used MGL Tools-based Autodock software between the selected compounds from *Saussurea lappa* and COX-2 receptor (PDB ID: 3NT1). The final result compared with the control of ligand Naproxen. Before starting the research, method validation was conducted to ensure data accuracy. For the receptors iNOS (PDB ID: 3E7G), the selected compounds were summarized from a previous study. LGA was used to score the molecules. After the molecular docking simulation process yielded binding energy information, the lowest energy of the complex evaluation used Discovery Studio (Biova) [33]. Tables 2 and 3 list the results of the molecular docking simulation between 10 compounds from *Saussurea lappa* and the COX-2 and iNOS receptors.

Table 2 Results of docking between chemical compounds from Quts al Hindi (*Saussurea lappa*) and cyclooxygenase receptors (COX-2)

No.	Compounds	Binding energy (ΔG) (kcalmol^{-1})
1.	Naproxen (native ligan)	-9.1
2.	Costunolide	-9.4
3.	Costic acid	-9.6
4.	Dehydrocostus_lacton	-7.9
5.	α-Cyclocostunolide	-7.2
6.	Rupestonic_acid	-7.7
7.	Beta-elemene	-7.5
8.	Beta-selinene	-8.6
9.	Arbusculin_A	-6.1
10.	Cnicothamno	-7.7
11.	Beta-sitosterol	-7.7

Table 3 Results of docking between chemical compounds from Quts al Hindi (*Saussurea lappa*) and iNOS

No.	Compounds	Binding energy (ΔG) (kcalmol^{-1})
1	Ethyl4-[(4-methylpyriclin-2Yl)Amino]piredirine-1-carboxylate	-7.3
2	Costunolide	-7.2
3	Costic acid	-7.1
4	Dehydrocostus_lacton	-7.1
5	α-Cyclocostunolide	-6.8
6	Rupestonic_acid	-6.5
7	Beta-elemene	-6.4
8	Beta-selinene	-6.2
9	Arbusculin_A	-6.3
10	Cnicothamno	-6.0
11	Beta-sitosterol	-6.1

Based on the results of molecular analysis of the chemical compound test ligand docking from *Saussurea Lappa*, of the ten tested ligands analyzed, ΔG values were in the range of from –6.1 to –9.6 kcal/mol, and 2 test ligands provided strong inhibitory activity on the target protein because they had lower ΔG value compared to the native ligand. The test ligands are costic acid –9.6 kcal/mol and costunolide 9.4 kcal/mol. Meanwhile, the naproxen comparator ligand has an activity value of ΔG –9.1 kcal/mol. The results of the docking analysis of the test ligands with iNOS showed that of the ten tested ligands, there was one ligand, namely costunolide –7.2 kcal/mol, which exerted an inhibitory activity on the target protein close to the ΔG value of the comparator ligand ethyl 4-[(4-methylpyriclin-2-Yl)Amino]pyredirine-1-carboxylate of –7.3 kcal/mol.

The chemical compound test ligand from Quts al Hindi (*Saussurea lappa*), which has the highest interaction affinity with the native ligand, was then visualized using Discovery Studio software to determine the interaction between the test ligand and amino acid residues from the receptor, as shown in Tables 4 and 5, as well as in Figs. 4, 5, 6, 7, and 8. The visualization results show that there is a bond between the test ligand and the receptor amino acid residue that forms hydrogen bonds.

Table 4 Visualization results of the interaction of the tested ligands and amino acid residues of the COX-2 target protein

Compounds	ΔG (kcal/mol)	Hydrogen bond	Amino acid
Native ligan	-9.1	TYR355	VAL349, LEU531, LEU359, ARG120, TYR355, VAL523, ALA527, LEU352, MET522, PHE518, TRP387, LEU384
Costunolide	-9.4	TRP387	VAL349, LEU531, LEU352, PHE518, VAL523, LEU384, PHE381, TYR385, TRP387, ALA527
Costic Acid	-9.6	ARG120, TYR355	ALA527, VAL523, VAL349, LEU352, ARG120, TYR355

Table 5 Visualization results of the interaction of the tested ligands and amino acid residues of the iNOS target protein

Compounds	ΔG (kcal/mol)	Hydrogen bond	Amino acid
Native ligan	-7.3	GLU377, TYR347	GLU377, TYR347, TRP346, ASP382, TRP372, PHE369, VAL352
Costunolide	-7.2		PRO350, TYR347, TYR373, ARG381, VAL352

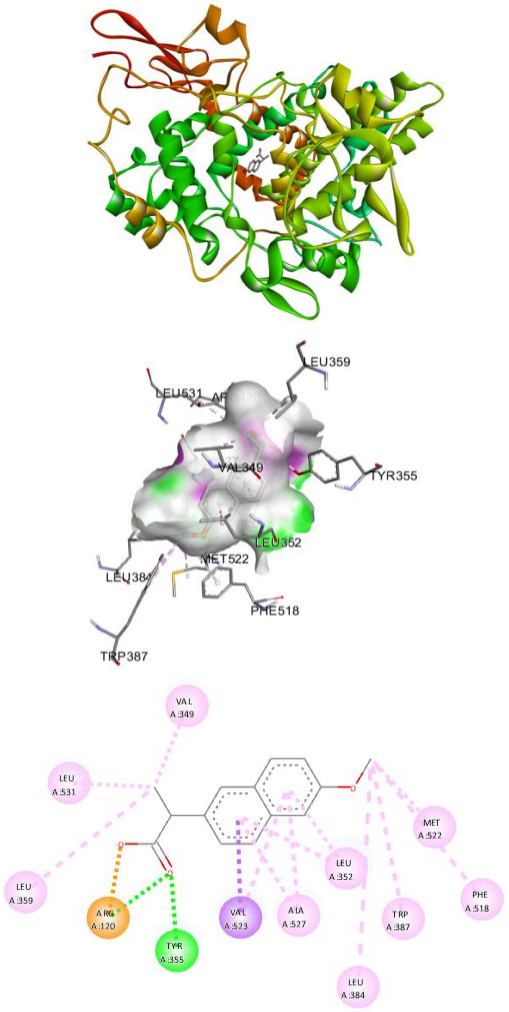
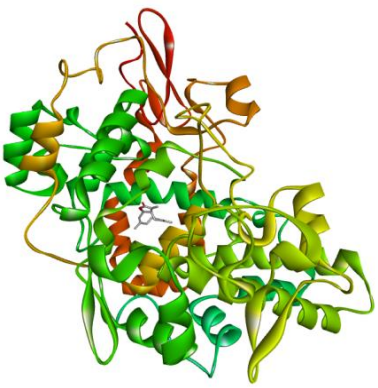


Fig. 5 Interaction of the COX-2 target protein with naproxen



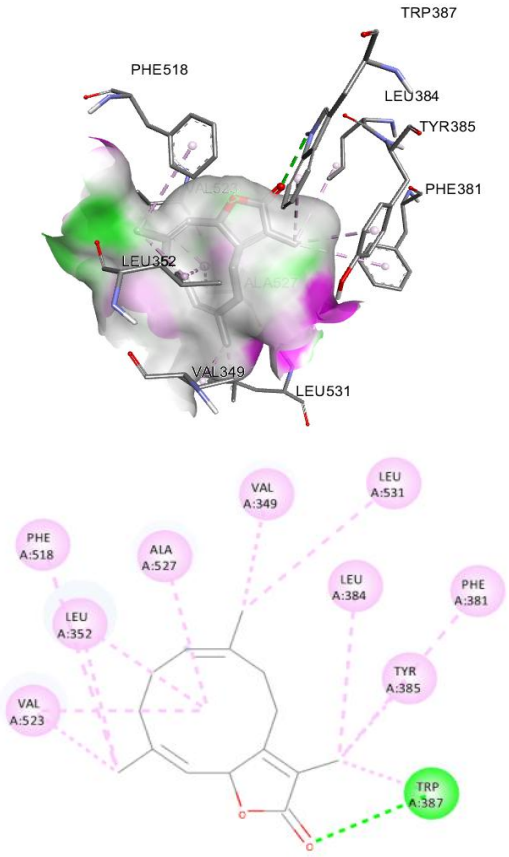
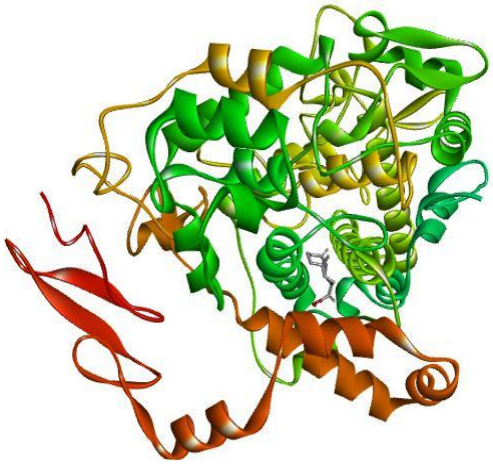


Fig. 6 Interaction of the COX-2 target protein with costunolide



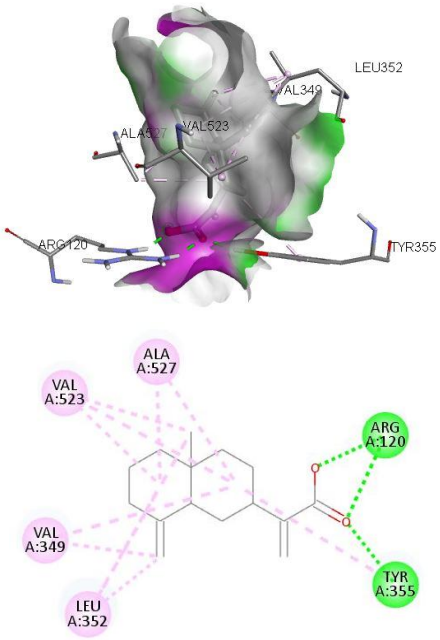


Fig. 7 Interaction of the COX-2 target protein with costic acid

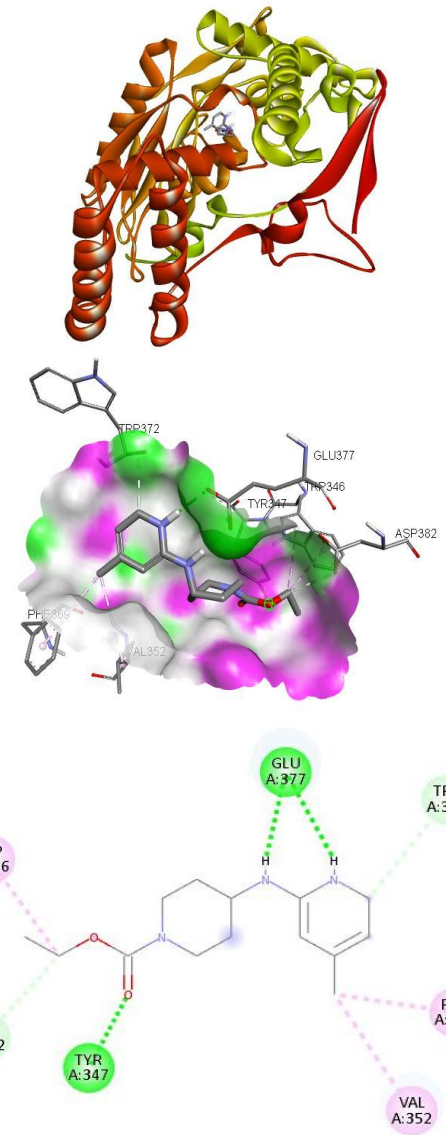
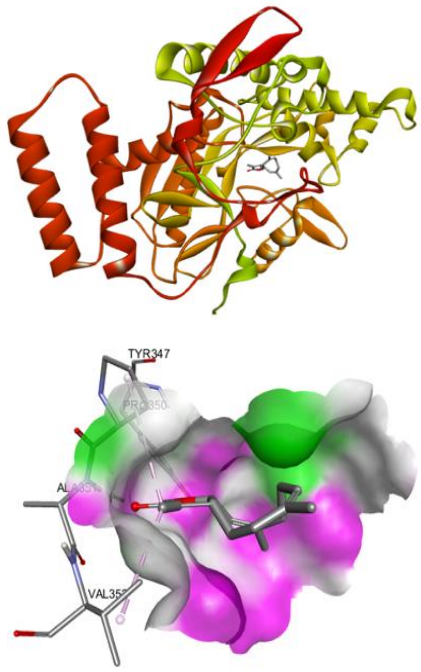


Fig. 8 Interaction of the iNOS target protein with (ethyl 4-[(4-methylpyriclin-2-Yl)amino]pyredirine-1-carboxylate)



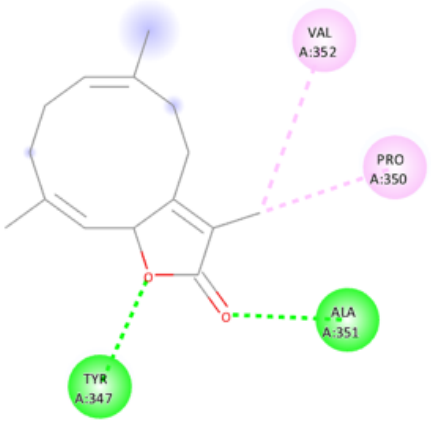


Fig. 9 Interaction of the iNOS target protein with costunolide

Based on the results of visualization of the interaction of the Naproxen comparison ligand with the COX-2 receptor, we can see that the Naproxen native ligand interacts with the receptor at the amino acid residues VAL349, LEU531, LEU359, ARG120, TYR355, VAL523, ALA527, LEU352, MET522, PHE518, TRP387, and LEU384, and there is one interaction through hydrogen bonding, namely on the TYR355 amino acid residue. The visualization of the interaction of the reference ligand Ethyl 4-[(4-methylpyriclin-2-Yl)Amino]pyredirine-1-carboxylate with the iNOS receptor interacts with the receptor on the amino acid residues GLU377, TYR347, TRP346, ASP382, TRP372, PHE369, and VAL352, and there are two interactions through hydrogen bonds, namely at GLU377 and TYR347. Binding to the amino acid residue indicates binding to the receptor binding site. Interactions through hydrogen bonds can stabilize ligand and receptor bonds and increase conformational stability [42, 43]. The amino acid residues of two ligands interacting with COX-2 and one ligand interacting with iNOS from the compound Quts al

Hindi (*Saussurea lappa*) were compared with the binding to the comparison amino acid residues Naproxen on COX-2 and ethyl 4-[(4-methylpyriclin-2-Yl)Amino]pyredirine-1-carboxylate on iNOS. The visualization results show that the amino acid residues of cosmic acid and costunolide are similar to the reference amino acid residues of the two target proteins with forming hydrogen bonds on these amino acid residues. Except for the costunolide ligand on the iNOS receptor, there are no hydrogen bonds, so the interaction can be predicted as less stable than other compounds.

3.2.2. Molecular Dynamics Test Results

A molecular dynamics simulation between the protein target COX-2 code (PDB 3NT1) and iNOS code (PDB 3E7G) with the best test ligand arising from the molecular anchoring simulation was conducted to observe the stability of the interaction between the protein and the ligand in a physiologically realistic manner. Before the molecular dynamics simulation, preparations were made, namely, preparing the topology and coordinates of the best test ligand, the COX-2 and iNOS receptor, and creating a complex between the test ligand and the receptor. Subsequently, the molecular dynamics simulation involved several stages, namely, the solvation, neutralization, minimization, equilibration, and production stages. The solvation stage was performed by dissolving the protein/ligand system in the TIP3P water model, while in the neutralization stage, Na^+ and Cl^- ions were added to make the system neutral (44). The minimization step helped to avoid inappropriate van der Waals contacts and to minimize the high-energy steric effects of the structure. Furthermore, the equilibration stage made it possible to set the system at a constant temperature, volume, and pressure (45, 46). Finally, the last stage allowed the molecular dynamics simulation to run for 100 ns. RMSD analysis made it possible to see and compare the conformational shift of a molecule during the simulation. This RMSD analysis was performed on the backbone of the complex, and Figs. 9 and 10 show its results.

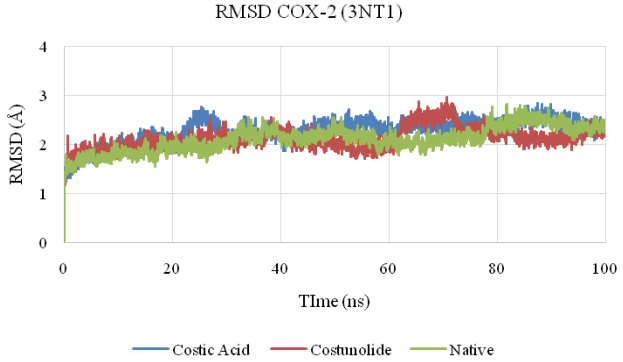


Fig. 10 RMSD values of cosmic acid (blue), costunolide (red), and native ligand COX-2 (green) as a function of time

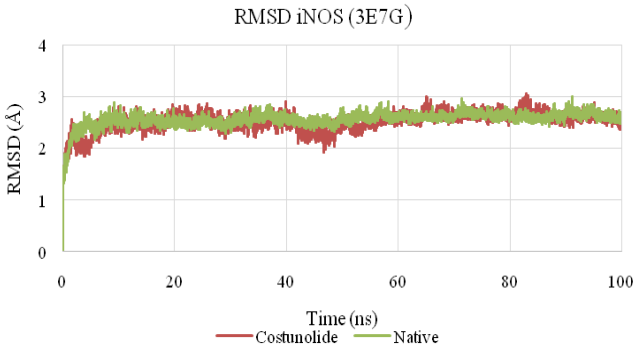


Fig. 11 RMSD values of the costunolide (red) and the native ligand iNOS (green) as a function of time

The simulation that lasted 100 ns showed that at the beginning, there was an increase in the RMSD backbone value of costic acid and costunolide (3–4 Å) that was higher than that of the native ligand COX-2 (2–2.5 Å) and the value of costunolide (2.5–3.5) that was higher than that of the native ligan iNOS (2.5–3 Å). The increase in the RMSD backbone value indicates that the enzyme structure begins to open, and the ligand searches for the appropriate binding site or coordinates with the protein conformation. The study of the native ligand system revealed that after a particular time, the system tends to be stable, but at the end of the simulation, its RMSD backbone value increases again. The stable RMSD backbone value indicates an interaction between the protein residues and the ligand, and as a result, the protein tends to maintain its structure. Although the costic acid and costunolide compound system at the beginning of the simulation appears stable, it then fluctuates for a particular time before increasing its RMSD backbone and, at the end of the simulation, starting to stabilize again.

The analysis involved producing an RMSF graph of the amino acid residues. The RMSF is a measure of the deviation between the position of the particle and some of its reference positions, and it is used to calculate the extent to which the fluctuation or movement of each residue occurs during the simulation; in fact, it describes the conformational shift of each amino acid residue that provides the protein with flexibility. Figs. 11 and 12 show the RMSF graph for both residues.

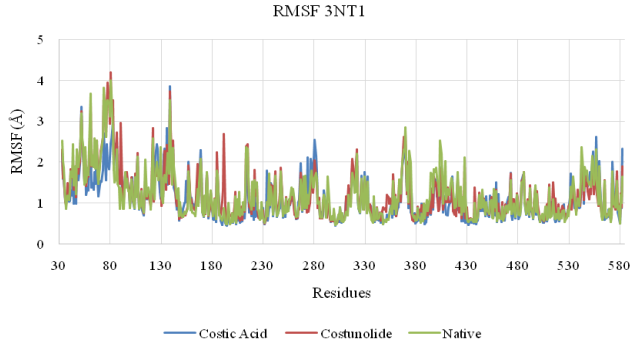


Fig. 12 RMSF values of costic acid (blue), costunolide (red), and native ligand COX-2 (green) as a function of time

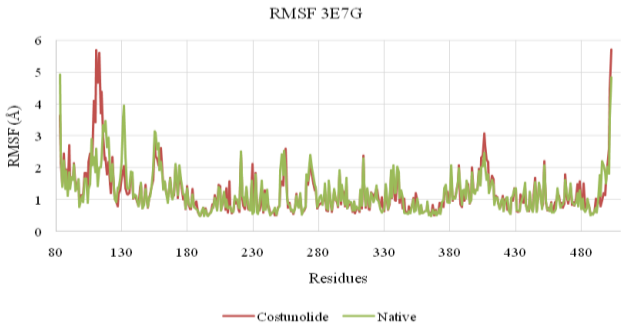


Fig. 13 RMSF values of costunolide (red) and native ligand iNOS (green) as a function of time

The results of the RMSF analysis showed that, overall, there was a similar flexibility on behalf of COX-2 and INOS regarding its binding to the native ligand with costic acid and costunolide. The amino acid residues with the highest fluctuation and flexibility against costic acid and costunolide (COX-2) were the amino acid residues 80, 132, 183, 280, and 550, and costunolide (iNOS) were the amino acid residues 100, 150, 250, 400, and 530. These residues demonstrated unstable interactions or bonds and experienced the most positional changes during the 100-ns molecular dynamics simulation. In contrast, the amino acid residues with low flexibility against costic acid and costunolide were generally the same as the interacting residues during the docking simulation. As a result, these residues are essential residues that form part of the COX-2 and iNOS active sites and mark the stability of the residue region that binds to costic acid and costunolide.

3.2.3. Prediction of Binding Energy by MM-PBSA

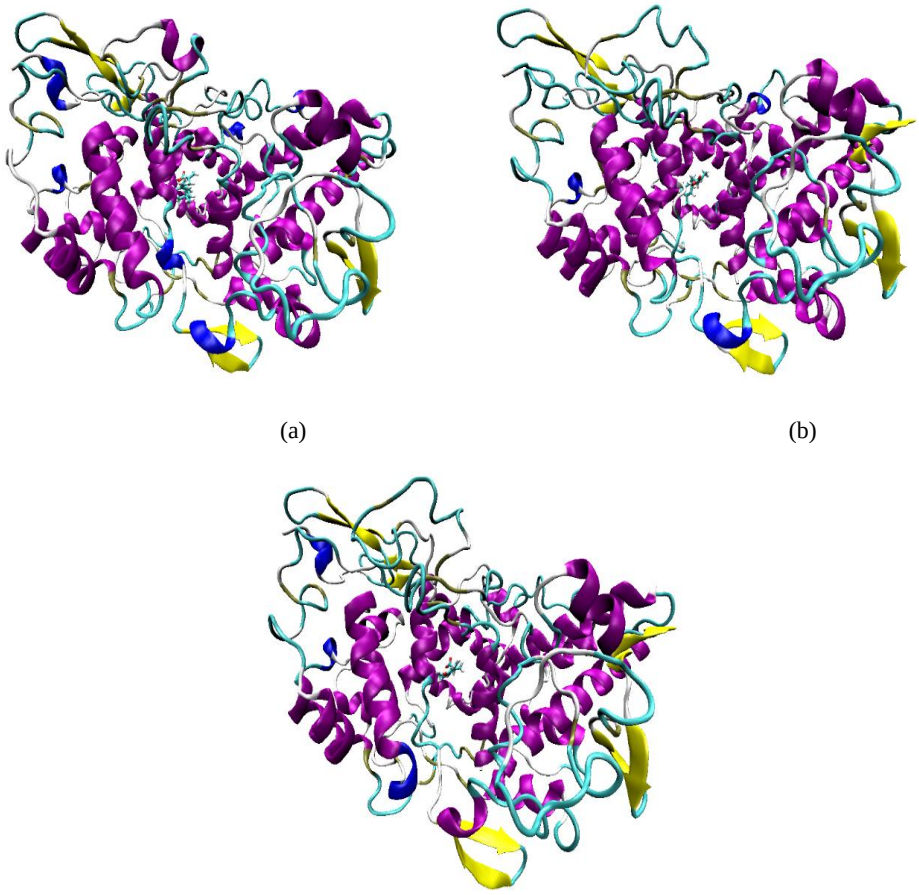
The binding free energy of the molecular dynamics trajectories calculation of the examined complex systems used the MM-PBSA method (Tables 6 and 7). Based on this calculation, the Van der Waals, electrostatic, and SASA energies in both systems demonstrated a negative value, whereas the polar solvation energy produced a positive value. These results indicate–in both systems–that the polar solvation energy terms opposed to the binding have favored the binding. This is because the polar solvation energy in the native ligand complex was more positive than that in the *Saussurea lappa* (polar solvation energy of the costic acid and costunolide was 78.004 ± 9.389 kJ/mol and 81.832 ± 13.393 kJ/mol, while the respective value of the native ligand COX-2 was 265.382 ± 18.818 kJ/mol) and costunolide was 65.933 ± 14.320 kJ/mol, while the respective value of the native ligand iNOS was 83.859 ± kJ/mol. The total binding free energy of the costic acid and costunolide complex had lower total bond energy, a fact that indicates that the level of affinity of *Saussurea lappa* (antagonist) is better than that of the native ligand (agonist) to COX-2 and iNOS. Figs. 13 and 14 present a visualization of the molecular dynamics simulation of costic acid, costunolide, and the native ligand on the active site of COX-2 and iNOS.

Table 6 The binding free energies were calculated using the MM-PBSA method (COX-2)

Compounds	Van der Waal	Electrostatic	Polar solvation	SASA	Binding energy
Costic Acid	-156.563 ± 9.236	-11.771 ± 4.705	78.004 ± 9.389	-15.326 ± 0.570	-105.656 ± 10.146
Costunolide	-144.030 ± 11.195	-3.420 ± 10.121	81.832 ± 13.393	-15.730 ± 0.630	-81.347 ± 12.351
Native	-131.808 ± 12.996	-260.041 ± 22.446	265.382 ± 18.818	-14.967 ± 0.591	-141.434 ± 12.946

Table 7 The binding free energies were calculated using the MM-PBSA method (iNOS)

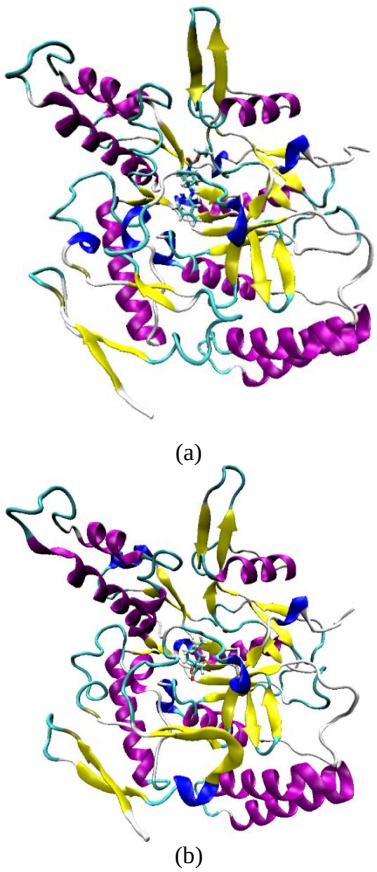
Compounds	Van der Waal	Electrostatic	Polar solvation	SASA	Binding energy
Costunolide	-107.808 ± 16.260	-23.144 ± 8.199	65.933 ± 14.320	-12.896 ± 1.159	-77.916 ± 19.447
Native	-117.050 ± 17.031	-23.320 ± 15.982	83.859 ± 24.678	-15.026 ± 2.223	-71.538 ± 16.456



(a) (b)

(c)

Fig. 14 Visualization of the molecular dynamics simulation of (a) costic acid, (b) costunolide,and (c) the native ligand on the active site of the COX-2 protein receptor



(a)

(b)

Fig. 15 Visualization of the molecular dynamics simulation of (a) costunolide and (b) the native ligand on the active site of the iNOS protein receptor

4. Conclusion

S. lappa exhibited membrane stabilization effects by inhibiting hypotonicity-induced lysis of erythrocyte membranes. The erythrocyte membrane is analogous to the lysosomal membrane, and its stabilization implies that the extract may also stabilize the lysosomal membrane. Stabilization of the lysosomal membrane is essential in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils, such as bacterial enzymes and proteases, which cause further tissue inflammation and damage. The above study concluded that the root extract of *Saussurea lappa* has significant membrane stabilization properties comparable to those of the standard drug diclofenac sodium. This assumes that the affinity of the compounds of *Saussurea lappa* is stronger than that of diclofenac sodium, so it is predictable that *Saussurea lappa* is more selective in inhibiting the COX-2 enzyme than the active site of the iNOS enzyme. Thus, Quts al Hindi extract containing the active compounds costic acid and costunolide can apply an anti-inflammatory agent. The limitations of this study were that the active compounds of *S. lappa* that have anti-inflammatory properties have not been isolated. Therefore, we can recommend further

research to isolate compounds in non-polar and semipolar solvent fractions.

Acknowledgment

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