IN-SILICO ANALYSIS OF EUGENOL AND BETA-CARYOPHYLLENE COMPOUNDS IN CLOVE (*Syzygium aromaticum* L.) ON NF-κB PROTEIN AS ANTI-INFLAMMATORY AGENT IN ATHEROSCLEROSIS

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Abstract: Atherosclerosis is one of the leading causes of cardiovascular disease, which is ranked as the world's deadliest disease by WHO. The NF-kB protein is important in the development of atherosclerosis. Inhibiting the inflammatory response pathway via the NF-kB protein can inhibit the development of atherosclerosis. Clove (*Syzygium aromaticum* L.) is one of the plants with anti-inflammatory and anti-atherosclerotic properties. The main constituents of clove are eugenol and beta-caryophyllene (BCP), which have been extensively researched for their anti-inflammatory properties. This study aims to simulate the potency of eugenol and BCP compounds by measuring their binding affinity and observing the interactions that occur when they are tethered to the active site of the NF-kB protein. The research was carried out in silico method, using molecular docking techniques. According to the analysis, eugenol and BCP had binding affinity values of -5.9 kcal/mol and -7.0 kcal/mol, respectively. The eugenol compound forms 12 interactions on the active site of NF-kB protein, consisting of the hydrogen bond, carbon-hydrogen bond, pi-sigma, alkyl, and Van Der Waals interactions. Meanwhile, 5 interactions form between BCP and NF-kB protein, including Van Der Waals, alkyl, and pi-sigma interactions. The bond affinity calculations of the eugenol-NF-kB protein are stronger than the BCP-NF-kB protein. In addition, the number of interactions formed by eugenol- NF-kB protein is greater than BCP-NF-kB protein. Based on the description, the eugenol compound has better potential to influence the function of the NF-kB protein than BCP, so the eugenol compound is recommended for in vitro and in vivo anti-inflammatory tests.
Keywords: atherosclerosis; anti-Inflammatory; clove (*Syzygium aromaticum* L.); molecular docking; NF-κB protein

Abstrak: Aterosklerosis adalah salah satu penyebab utama penyakit kardiovaskular, yang menduduki peringkat penyakit paling mematikan di dunia menurut WHO. Protein NF-kB penting dalam perkembangan aterosklerosis. Menghambat jalur respon inflamasi melalui protein NF-kB dapat menghambat perkembangan aterosklerosis. Cengkih (Syzygium aromatikum L.) merupakan salah satu tanaman yang mempunyai sifat anti inflamasi dan anti aterosklerotik. Kandungan utama cengkeh adalah eugenol dan beta-caryophyllene (BCP), telah banyak diteliti sifat anti inflamasinya. Penelitian ini bertujuan untuk mensimulasikan potensi senyawa eugenol dan BCP dengan mengukur afinitas pengikatannya dan mengamati interaksi yang terjadi ketika ditambatkan pada sisi

aktif protein NF-kB. Penelitian dilakukan secara in silico dengan menggunakan teknik molekuler docking. Berdasarkan analisis, eugenol dan BCP memiliki nilai afinitas pengikatan masing-masing sebesar -5,9 kkal/mol dan -7,0 kkal/mol. Senyawa eugenol membentuk 12 interaksi pada sisi aktif protein NF-kB, terdiri dari interaksi ikatan hidrogen, ikatan karbon-hidrogen, pi-sigma, alkil, dan Van Der Waals. Sementara itu, terjadi 5 interaksi antara protein BCP dan NF-kB, antara lain interaksi Van Der Waals, alkil, dan pi-sigma. Perhitungan afinitas ikatan protein eugenol-NF-kB lebih kuat dibandingkan protein BCP-NF-kB. Selain itu, jumlah interaksi yang terbentuk protein eugenol-NF-kB lebih besar dibandingkan protein BCP-NF-kB. Berdasarkan uraian tersebut, senyawa eugenol mempunyai potensi yang lebih baik dalam mempengaruhi fungsi protein NF-kB dibandingkan BCP, sehingga senyawa eugenol direkomendasikan untuk uji antiinflamasi dengan metode in vitro dan in vivo.

Kata kunci: aterosklerosis; anti-Inflamasi, cengkeh (*Syzygium aromaticum* L.), penambatan molekuler, protein NF-κB

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Introduction

Atherosclerosis is a chronic inflammatory disease caused by an injury to the artery's inner wall (intima). These injuries occur due to various risk factors such as hyperlipidemia, hypertension, cigarette smoke toxins, genetics, or due to an unhealthy lifestyle (Erizon and Karani, 2020; Peñalvo et al., 2021; Singh et al., 2021). This disease is caused by endothelial dysfunction, which is accompanied by lipid retention in the arterial wall's intima (Aziz and Yadav, 2016; Manggasa, 2017).

The injured endothelial wall will then be activated, resulting in the release of inflammatory cytokines. P ro-inflammatory cytokines such as tumor necrosis factor (TNF-) and interleukin-1 (IL-1) will activate the transcription factor NF-B (nuclear factor-kappa beta), causing an increase in the production of inflammatory cytokine molecules such as expression of adhesion molecules which include vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (Pamukcu et al., 2011; Erizon and Karani, 2020). These adhesion molecules will initiate an inflammatory response by attracting inflammatory cells like monocytes and T cells into the intima of the arterial wall (Aziz and Yadav, 2016).

Monocytes that have already entered the intima will differentiate into macrophages, which will then consume oxidized lipids (ox-LDL) to form foam cells (Qahtany et al., 2018; Candra and Wijaya, 2021). Sustained accumulation of foam cells will result in the formation of a fatty streak in the inflamed area, which will gradually thicken and form a fibrous cap, also known as plaque (Aziz and Yadav, 2016).

The NF-B protein is important in inflammatory diseases such as atherosclerosis because it is the key to the immune response (Barboza et al., 2018). Inhibiting the activity of the NF-kB transcription factor has been shown to reduce the expression and release of inflammatory mediators such as monocytes, macrophages, T cells, B cells, and endothelial cells, suggesting that NF-kB may be a target for anti-inflammatory therapy (Pamukcu et al., 2011). Susanti et al. (2018) investigated the effects of cyanidin and peonidin compounds from purple sweet potato plants on the NF-κB protein in silico, and the results indicated a high potential for inhibiting atherogenesis. The binding affinity values for cyanidin and peonidin were -7.92 kcal/mol and -7.86 kcal/mol, respectively. Based on this, the authors are interested in finding other bioactive compounds as anti-inflammatory agents to prevent the formation of atherosclerotic plaques.

Eugenol and beta-caryophyllene (BCP) are the major compounds contained in the clove plant (Syzygium aromaticum L.). The percentages are respectively 70-85% and 5-12% (Mittal et al., 2014). Eugenol is a compound that belongs to the phenol group. This compound has several functional groups in the form of allyl (-CH_2-CH=CH_2), phenol (OH), and methoxy (-OCH_3) which allow eugenol to be the basic material for the synthesis of various other compounds with higher values such as isoeugenol, eugenol acetate, vanillin, and others. For its utilization, eugenol compounds, and their derivatives are generally used in various industries such as the food industry, pesticides, cosmetics, pharmaceuticals, and other chemical industries (Jufri, 2010; National Center for Biotechnology Information, 2021). BCP is a sesquiterpene compound found in many natural products such as clove oil, cinnamon leaves, and copaiba balsam. This substance can be used as a natural medicine as well as a fragrance (Jufri, 2010). The formation of macromolecular derivatives of eugenol as well as the latest developments and further perspectives in their pharmacological and antimicrobial applications (Kowalewska, 2023).

Many studies have been conducted to investigate the anti-inflammatory potential of eugenol and BCP. Eugenol has anti-inflammatory and immunomodulatory properties that can inhibit the NF-kB pathway, lowering the risk of various cardiovascular disorders (Batiha et al., 2020). Similarly, BCP compounds are capable of inhibiting NF-kB, which is one of the main mediators of an inflammatory response (Scandiffio et al., 2020). As a result, an in silico test of eugenol and BCP compounds on NF-kB was performed in this study using the molecular docking method. The purpose of the study was to determine their binding affinity and their interactions with the NF-kB protein. The potential and effectiveness of eugenol and BCP in inhibiting inflammation in atherosclerosis can be predicted in this manner.

Materials and Methods

Materials

The 3D structure of the NF-B protein and its native ligands, as well as the 3D

structure of eugenol and BCP compounds from clove, were used in the study (Syzygium aromaticum L.). The structure of the NF-kB was obtained from the Protein Data Bank (PDB) website at www.rcsb.org/pdb. by typing the NF-kB protein ID, 4IDV, in the search box (Susanti et al., 2018; Candra and Wijaya, 2021). the file was downloaded and saved in ".pdb" format. The 3D structures of the eugenol and beta-caryophyllene were obtained from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) by typing the compound name in the search box (Sholihah et al., 2015; Varghese et al., 2017). The files of these two compounds were downloaded in the format ".sdf" and the format was changed to ".pdb" by using the Discovery Studio Visualizer program (Alifah, 2019).

Equipment

A computer with Windows 10 64-bit, the SwissADME program (www.swissadme.ch), the Pyrx 0.8 program, and the BIOVIA Discovery Studio Visualizer.

Methods

Identification of NF-kB Active Sites: Protein active sites were identified using 3D crystallographic structures of NF-kB proteins downloaded from PDB sites. The active site of the protein is viewed using the Hierarchy Tool in the DSV program and then used as a reference for setting the grid box for the redocking stage of the default ligand and the docking stage of the test compounds (Musfiroh et al., 2020).

Similarity Analysis of Test Compounds: The drug similarity analysis of the test compounds eugenol and beta-caryophyllene in clove (Syzygium aromaticum L.) was performed using the ADME analysis service available on the website www.swissadme.ch. The Canonical SMILES formula for each compound obtained from PubChem was pasted into a special box on the SwissADME website (Alifah, 2019). If the results meet the majority of Lipinski's five rules, the research is continued to the next stage.

Preparation of Target Receptor: The A chain of the NF-kB protein was used as the receptor. At this stage, The DSV program was used to separate it from other chains and water molecules. Following that, A chain of the NF-kB was separated from its default ligand to create a space (cavity/pocket) for the test compound in the docking process (Susant, et al., 2015).

Preparation of Test Ligands: The 3D structures of the innate ligands, eugenol compounds, and BCP compounds were prepared using the Pyrx 0.8 program before the validation and docking of the test compounds. To avoid unwanted interactions (bad contact), the energy of the three test ligands was then minimized using the Open Babel feature (Noviardi, 2010).

Validation of molecular docking: The validation stage was completed by reattaching the default ligand to NF-kB (Candra and Wijaya, 2021). The grid box was determined by the active side obtained in the previous stage. After the docking process was finished, the best pose with the most negative binding affinity value and the lowest bound RMSD value <3 A was selected.

Connecting the Test Compound to the Target Receptors: The eugenol and beta-caryophyllene test compounds were docked one by one in the same manner as the validating method (Susanti et al., 2018).

Visualization of Molecular Docking Results: The DSV program was used to visualize the results of molecular docking of the test compounds (Febrian, 2018). The interaction between the test ligand and the target receptor was then observed and analyzed.

Data Analysis: The data from molecular docking were analyzed using the parameters of the lowest binding affinity value, RMSD lower bound with a value of < 3 Å, and the number of interactions formed between the tested ligands and the active site of the NF-κB (Susanti et al., 2018; Amarawati et al., 2019). The formed interactions are visualized using 2D and 3D visualizations projected by the DSV.

Results and Discussions

Similarity Analysis of Tested Compounds

Eugenol and BCP meet the five Lipinski rules, with a molecular weight ≤ 500 dalton, total log-p <5, hydrogen donors, 5, acceptor hydrogens, 10, and molar refractivity values ranging from 40 to 130. At this point, it can be concluded that eugenol and BCP compounds derived from clove can be used as drug candidates because they produce positive results, allowing the research to progress to the next stage.

According to Putra et al. (2020), an oral drug with a molecular weight of fewer than 500 daltons is easily absorbed by the body. Meanwhile, if the molecular weight is greater than 500 daltons, absorption will be reduced due to a reduction in the concentration of molecules on the surface of the intestinal epithelium. (Yasin et al., 2020).

The higher the log-p value, the greater the hydrophobicity of a tested molecule and the interaction between the molecule and the target receptor takes longer. Because the drug compounds are retained by the lipid bilayer membrane, resulting in the compounds being distributed in the body and reducing the selectivity of the compounds (Noviardi, 2010).

The number of donor and acceptor hydrogen atoms in a ligand-receptor bond indicates the energy level required to complete the absorption process. The greater the hydrogen capacity formed, the greater the energy required. The total number of donor and acceptor hydrogens in a candidate molecule cannot exceed 5 and 10, respectively (Rachmania et al., 2018).

The molar refractivity value should not be greater than 40-130 because it is related to the polarizability of the molecule. The solubility of a compound in water is determined by its polarization ability. If the polarizability of a molecule is high, the resulting bonds with other molecules are also generally strong (Daddam et al., 2020).

Table 1. The results of Lipinski analysis using the SwissADME site

Parameter	Innate Ligand	Eugenol	BCP
Molecular weight (g/mol)	366,41	164,20	204,35
Log-P value	2,13	2,25	4,24
Hydrogen Donor	3	1	0
Hydrogen Acceptor	5	2	0
Molar refractivity	104,48	49,06	68,78

Identification of Active Sites and Preparation of NF-κB as Receptors

The crystallographic structure of a target receptor recorded in the Protein Data Bank is one way to identify its active site (PDB). The DSV program can read the active site of the target receptor and mark it by highlighting the active site.

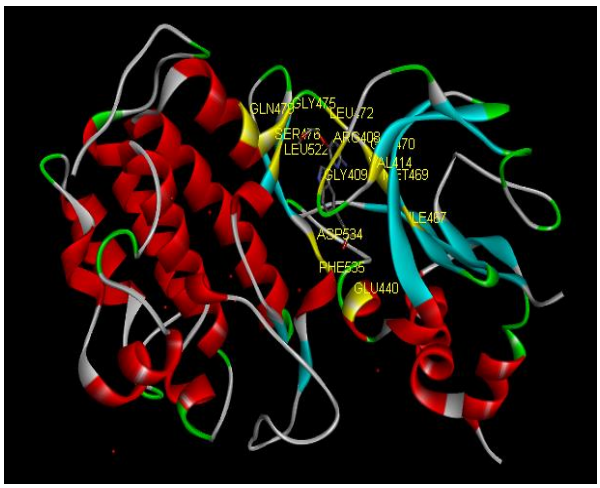


Figure 1. The position of the active site of NF-Kb on Chain A is markered by yellow color

DSV reads 14 residues in the NF-B protein that are predicted to be the protein's active site. The residues consist of Arginine 408, Glycine 409, Valine 414, Glutamic Acid 440, Isoleucine 467, Methionine 469, Glutamic Acid 470, Leucine 472, Glycine 475, Serine 476, Glutamine 479, Leucine 522, Aspartic Acid 534, and Phenylalanine 535. The active site of the NF-B protein is located within the secondary structure of the A chain, as shown in Figure 1.

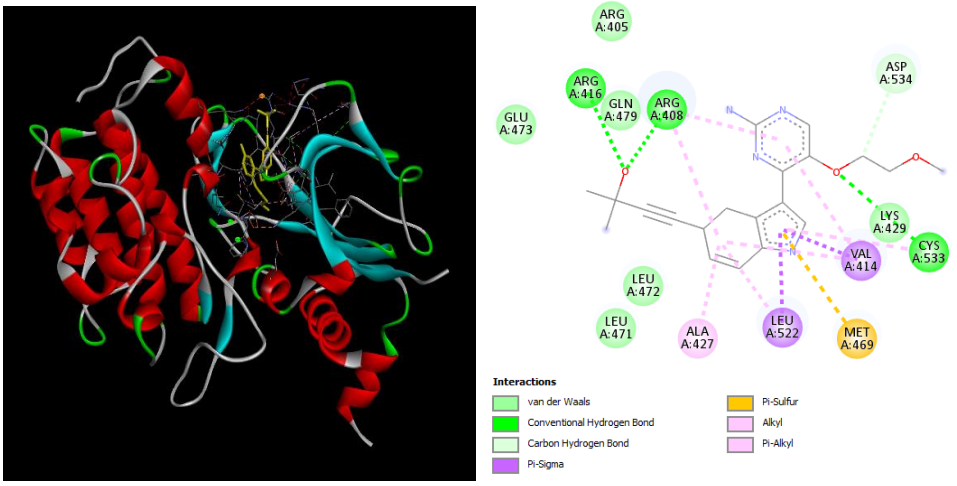
The A chain in the NF-kB protein was carried out because this protein has four identical chains, namely the A, B, C, and D chains (Susanti et al., 2018). The

four chains form a functional quaternary structure with four similar ligands, allowing one of the chains can represent the overall protein structure.

In the following step, water molecules are removed from the structure of the NF-kB so that the binding process between the active site of the NF-kB and the test compound is not hampered. Finally, proteins are separated from their default ligands to provide a cavity or pocket for the test compound in the docking process (Susanti et al., 2015). This process also yielded the innate ligand of the NF-kB, which will be used in the validation stage, namely 4-3-[2-amino-5-(2-methoxy ethoxy)pyrimidin-4-yl]. PubChem ID: 13V for -1H-indole-5-yl-2-methylbut-3-yn-2-ol (Candra and Wijaya, 2021).

Validation of Molecular Docking Method

Validation was performed by reattaching the default ligand to NF-kB, and the binding affinity and RMSD values were -9.3 kcal/mol and 2.095, respectively. According to Alifah (2019), the more negative the binding affinity value (away from zero), the stronger the bond formed between the ligand and its target receptor. Meanwhile, Ramrez and Caballero (2018) state that the docking method is acceptable or valid if the RMSD value is in the range of 2-3 Å. This statement implies that the method used in this study is valid so that the research can be continued to the next stage. The results of this validation will then be used as a positive control for the docking of eugenol and BCP compounds in the next stage.



a b

Figure 2-a. the result of 3D visualization of validationm ethod results; **Figure 2-b.** 2D visualization of the validation method. The interactions are shown by dashed lines

Figures 2-a and 2-b are 3D and 2D visualizations of predicted ligand-receptor interactions between innate ligands and the NF-kB protein, respectively. In total, 21 interactions were formed, with 7 different types of interactions. These

interactions include 2 types of hydrogen interactions with a total of 4 bonds, 3 types of hydrophobic interactions with a total of 10 bonds, 6 types of van der Waals interactions, and 1 other interaction in the form of a pi-sulfur bond. Interactions that form hydrogen bonds are indicated by green and light green colors. Hydrophobic bonds are indicated by purple and pink colors, Van der Waals bonds are indicated by cyan colors and sulfur bonds are indicated by orange colors.

The interactions formed reveal that the majority of the residues involved act on the active site of the NF-kB protein. The residues include Arginine 408, Valine 414, Methionine 469, Leucine 472, Glutamine 479, Leucine 522, and Aspartic Acid 534. Arginine 408 residue forms three types of bonds: conventional hydrogen bonds, alkyl bonds, and pi-alkyl bonds. Valine 414 residue also forms three types of bonds: pi-sigma, alkyl, and pi-alkyl. Methionine 469, Leucine 472, Glutamine 479, and Aspartic Acid 534 form one type of bond: pi-sulfur, Van Der Waals, and carbon-hydrogen bonds.

Leucine 522 forms three types of bonds: two pi-sigma bonds and one alkyl bond. In total, 13 bonds occur in the active site of the NF-κB protein. The residues and interactions can be seen in Table 2 The number of interactions formed on the active site of the NF-kB protein supports the validity of the method used. The number of interactions that occur between a ligand and amino acids on the active site of a protein, as mentioned by Ragi et al. (2021) is also a determining factor for whether a ligand/inhibitor is good or not.

Table 2. Residual validation results that act on the active site on NF-kB protein

Name of Residue	Number of interaction	Type of Interaction
Arginine 408	3	Conventional hydrogen, alkyl, pi-alkyl
Valine 414	3	Pi-sigma, alkyl, pi-alkyl
Methionine 469	1	Pi-sulfur
Leucine 472	1	Van der Waals
Glutamine 479	1	Van der Waals
Leucine 522	3	Pi-sigma, pi-sigma, alkyl
Aspartic acid 534	1	Hydrogen carbon

Analysis of Molecular Binding of Test Compounds to NF-κB Protein

Molecular docking simulations of eugenol and BCP to the NF-kB protein were performed using the Pyrx 0.8 program's AutoDock Vina feature. As was done during the validation stage, the grid box settings are adjusted to the previously known active sites. The best conformation with the most negative binding affinity value was selected from among the nine produced.

The results of the docking of the two compounds can be seen in Table 3.

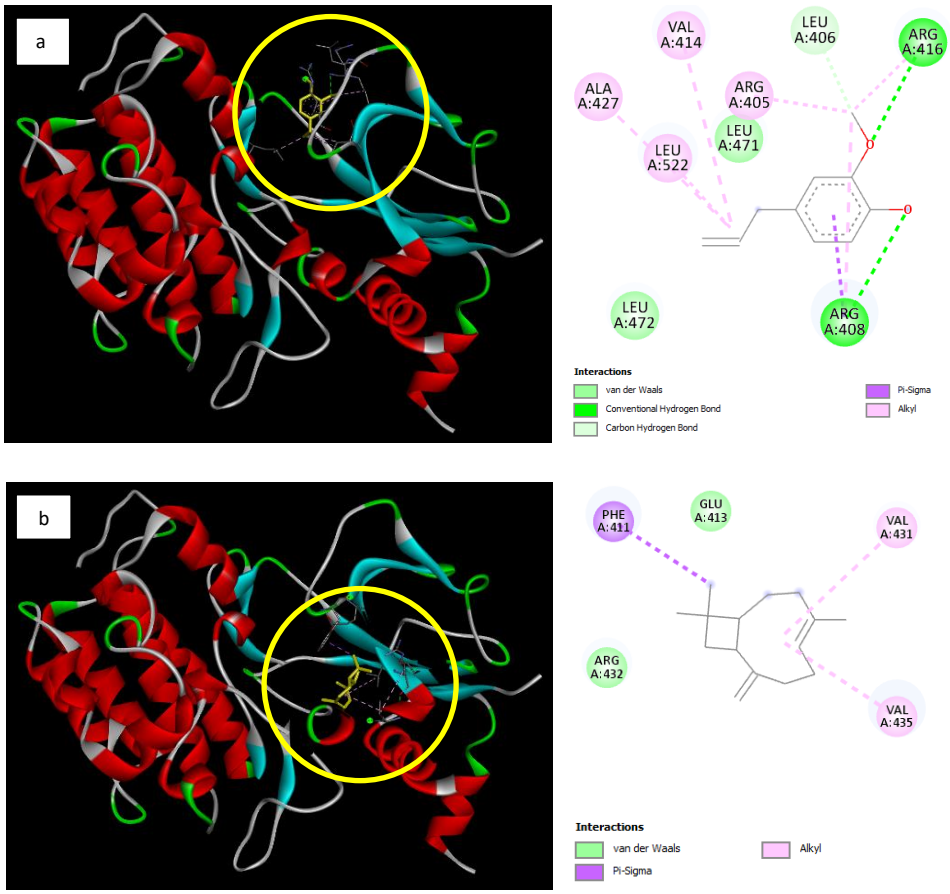


Figure 3. Interaction of eugenol (a) and BCP (b) with NF-κB protein. The yellow circle indicates the binding location of the test compound. The image on the left shows a 3D visualization, and the image on the right shows a 2D visualization.

Table 3 shows a comparison of binding affinity values and RMSD values between the three ligands tethered to the NF-κB protein, namely the innate ligand, eugenol compound, and BCP compound. The binding affinity values of the three tested compounds were respectively -9.3 kcal/mol, -5.9 kcal/mol, and -7.0 kcal/mol. Based on these findings, the two major clove plant compounds tested in this study performed no better than the innate ligand NF-B protein in terms of binding. Meanwhile, the bonds between eugenol and BCP appear to be more stable and spontaneous because they have a lower binding affinity value (Sholihah et al., 2015).

Table 3. The results of molecular docking of tested compounds on NF-kB

Tested Ligand	Binding Affinity	RMSD
Innate ligand (positive control)	-9,3 kcal/mol	2,095 Å
Eugenol	-5,9 kcal/mol	1,053 Å
BCP	-7,0 kcal/mol	1,168 Å

Although the BCP appears to have a higher binding affinity than the eugenol, more residues interact with the eugenol than the BCP. As shown in Figure 3, eugenol forms 12 bonds, which include two conventional hydrogen interactions (Arginine 408, Arginine 416), one carbon-hydrogen bond (Leucine 406), two types of hydrophobic interactions (Arginine 408), six alkyl bonds (Arginine 405, Arginine 408, Valine 414, Arginine 416, Alanine 427, Leucine 522), and two Van Der Waals interactions (Leucine 471, Leucine 472). Meanwhile, the BCP only forms 5 interactions, including 3 hydrophobic interactions in the form of a pi-sigma bond (Phenylalanine 411) and two alkyl bonds (Valin 431, Valin 435), as well as two Van Der Waals interactions (Glutamic Acid 413, Arginine 432).

Of the 12 interactions formed in the eugenol, there are 4 residues out of a total of 9 residues that bind to the eugenol, acting on the active site of the NF-κB protein. These residues are arginine 408, Valine 414, Leucine 472, and Leucine 522. Meanwhile, the residues formed in the BCP compound appear to be outside the active site of the NFk-B protein, and there are no residues that are identical to the residues on the active side of the NF-kB protein. In terms of the number of interactions formed and the location of their binding, eugenol has better performance than BCP. As previously stated, the number of interactions in the ligand-receptor interaction is one of the parameters used to determine the potency of the drug (Ragi et al., 2021). The binding affinity value of BCP is more negative than eugenol, indicating that the affinity of BCP for amino acids outside the active site is much more stable and spontaneous, and thus this location may also have the potential to become a binding site or a new binding site. This is because a compound molecule will seek the most stable conformation in the active site of the target protein (Candra and Wijaya, 2021).

Hydrogen, electrostatic, and Van Der Waals interactions can stabilize a ligand-receptor bond (Rachmania, 2019). Hydrogen bonds form due to the attractive force between dipoles with two electric charges and when N, O, or F atoms in a molecule have lone pairs of electrons (Roni and Legiso, 2021). Electrostatic interactions, on the other hand, are weak non-covalent interactions that occur between atoms with different polarities. Although this interaction is weak, the presence of many electrostatic interactions can help the protein conformation become more stable (Rachmania, 2019). Van der Waals interactions are also relatively weak because these interactions can form between charged and uncharged amino acids. This is due to the permanent polarity of the molecule or because the molecule is induced (Arwansyah et al., 2014; Harnis et al., 2020).

Aside from these three types of interactions, hydrophobic interactions can form to stabilize the ligand-receptor complex. According to Rollando (2017), this interaction occurs in response to a decrease in free energy as environmental entropy increases. The presence of two non-polar groups, such as a lipophilic amino acid group in a ligand and a non-polar group in a receptor, each surrounded by a water molecule, is responsible for the increase in entropy. The two groups move closer to

each other and disrupt the surrounding water molecules trying to join with other water molecules.

The majority of the interactions formed in this study were hydrophobic and Van Der Waals interactions (VDW). Table 4 contains information on the number of interactions between these two groups.

Table 4. Comparison of the number of hydrophobic bonds and VDW test ligands

Test Ligans	Hydrophobic	VDW
Innate ligan (positive control)	11	6
Eugenol	7	2
BCP	3	2

According to the explanation above, the binding of eugenol with NF-kB protein indicates that this compound has the potential to inhibit NF-B protein activation and prevent the development of atherosclerotic plaques although it is not as effective as natural compounds in previous studies. The low binding affinity value and the large number of residues acting on the active site of the NF-B protein demonstrate this potential. While the BCP has a higher binding affinity than the eugenol, the lack of interactions formed on the active site of the NF-kB protein makes this compound unable to be considered as an inhibitor of the NF-kB protein, and therefore the activity of this compound needs to be further studied.

Conclusion

The binding affinity values of eugenol and BCP determined in this study were -5.9 kcal/mol and -7.0 kcal/mol, respectively. These two compounds had no more negative effects than the NF-kB protein-bound ligand, which was -9.3 kcal/mol.

The interactions formed from the molecular docking of the test compound with the NF-κB protein, it was found that the eugenol compound formed 12 interactions consisting of conventional hydrogen, carbon-hydrogen, pi-sigma, alkyl, and Van Der Waals interactions. Four of the 12 interactions work on the active site of the NF-kB protein. The BCP compound, on the other hand, forms 5 interactions with residues that bind outside the active site of the NF-kB protein.

Based on the value of binding affinity and the interactions formed in eugenol and BCP, eugenol is more effective than BCP as an inhibitor of NF-kB protein in preventing the development of atherosclerotic plaques. B CP compounds cannot be considered NF-kB protein inhibitors because no residues act on the active site of NF-kB protein.

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