Toxicity and molecular docking approach phenolic extract of Crescentia cujete L against the enzymes glutathione peroxidase and cathepsin K

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ABSTRACT

Osteoporosis is a metabolic bone disease characterized by a decrease in bone mass, caused by a reduction in bone matrix and minerals accompanied by microarchitectural deterioration of bone tissue. Factors such as hormone production, the aging process, and the formation of free radicals due to oxidative stress by Reactive Oxygen Species (ROS) can stimulate bone resorption. The content of secondary metabolite compounds in Crescentia cujete L. has medicinal properties for diseases. Purpose: this study was to determine the phenolic compound of Crescentia cujete L which has acted as an anti-osteoporosis with the approach of docking in silico. Extraction and partitioning of Crescentia cujete L fruit extracts in this study used n-hexane and toluene solvents with the aim of obtaining phenolic group compounds, knowing the toxicity value of partition results, obtaining the structure of phenolic compounds that have the potential to be anti-osteoporosis, and validating the ability of these phenolic compounds as anti-oxidants. As a result, n-hexane and toluene extracts are known to contain elevated levels of active and toxic compounds with LC50 values of 31.79 ppm and 93.49 ppm, respectively. The phytochemical test shows the presence of phenolic groups with maximum wavelengths at 302-320 nm. Benzene acetate, trans-cinnamic acid, and propanoic acid have the lowest affinity bonds with receptor 2F8A (Glutathione peroxidase); -4.21, -4.81, and -4.72 kcal/mol. Whereas at the 3KWZ receptor (Cathepsin K); -6.0, -5.4, and -5.0 kcal/mol.

Conclusion: The phenolic compound of Crescentia cujete extract L can be used as an alternative treatment for osteoporosis by inhibiting the enzymes Glutathione peroxidase and Cathepsin K.

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1. Introduction

Osteoporosis is a metabolic bone disease characterized by decreased bone mass, due to reduced bone matrix and minerals accompanied by damage to the microarchitecture of bone tissue, resulting in a tendency for bones to break easily [1]. Cellular osteoporosis is caused by the number and activity of osteoclast cells exceeding the number and activity of osteoblast cells (bone-forming cells).

More brittle and thin bones can also be caused by hormone production, the aging process, and the formation of free radicals due to oxidative stress by Reactive Oxygen Species (ROS) which can stimulate bone resorption [2]. Oxidative stress is the result of excess production of ROS in the body. ROS contain one or more unpaired electrons so it is very reactive to stabilize the electron balance. ROS production increases with age and some chronic diseases including osteoporosis.

Antioxidants are chemical compounds that can donate one or more electrons to free radicals. Free radicals are highly reactive and capable of oxidizing biological compounds such as carbohydrates, DNA, lipids and proteins. Antioxidants are divided into synthetic antioxidants and natural antioxidants based on the source of their acquisition [3,4].

The decrease in bone mass in osteoporosis sufferers is related to the process of bone remodeling, this includes two activities, namely: the process of bone resorption followed by the process of bone formation, the first process is known as osteoclast activity while the second is known as bone formation. osteoblasts. An imbalance in the number of osteoblasts and osteoclasts can be a major cause of osteoporosis. So when the formation of osteoclasts is inhibited, the resorption process can be reduced so that it will inhibit the osteoporosis process [5]. Treatment of osteoporosis depends on the severity, some drugs such as bisphosphonates, monoclonal antibodies, and also hormone therapy are aimed at increasing bone density.

Currently, a developing computational methods aimed at predicting the binding of a drug compound to a target protein or enzyme associated with a disease is Insilico molecular docking. Molecular docking is a genetic-based method based on the most appropriate interaction pattern through the entanglement of two molecules, namely the receptor and the ligand [6,7]. Ligands are organic or metallic molecules that are involved in both inorganic and biochemical processes [8]. Molecular docking approach is aimed at mimicking the interaction of a molecule with a protein that is the target of a metabolic mechanism or a target in in-vitro tests [9]. The main purpose of this docking is to achieve conformation optimal protein and ligand. The first step in molecular docking in computer-aided drug design is finding the ligand-binding site of a protein, where this ligand-receptor binding will affect the performance of the protein in the body’s metabolism.

Berenuk (Crescentia cujete L.) has been known as a medicine to treat several diseases caused by microorganisms. Berenuk fruit flesh is usually used by the community to treat diarrhea, stomach ailments, flu, bronchitis, coughs, asthma, urethritis, expectorants, antitussives, and laxatives [10,11]. The presence of phytochemicals such as saponins, flavonoids, cardenolides, tannins and phenols which are antioxidant compounds and the presence of hydrogen cyanide were present in the Berenuk fruit samples. Findings on phytochemical constituents, mineral composition and composition of Berenuk indicate that the fruit can make beneficial contributions to human and animal nutrition and can be used as medicine [12]. Therefore, research on the flesh of Berenuk (Crescentia cujete) which has antioxidant compounds belonging to the flavonoid group was carried out as an anti-osteoporosis.

2. Materials and Methods

This research was conducted at the Basic Chemistry laboratory of FT Untirta and the GC-MS test was carried out at the DKI Jakarta Provincial Health Laboratory which took place from March to September 2020. The tools used are a set of glassware, Rotary Evaporator, ultra sonicator weighing, GC-MS Agilent Technologies 7890, Laptop 8GB SSD 125, Autodock Tools Version 1.5.6 Sep_17_14, Biovia Discovery Studio Visualizer v21.1.0.20298, ChemOffice Professional Version 15.1.0.144, Materials used: bay leaf simplicia, technical ethanol, n-hexane, ethyl acetate, salmonella typhi bacteria, a set of MIC test media and culture, H2 gas, Argon gas, O2 gas.

2.1. Toxicity Test (BSLT-Brine Shrimp Lethality Test)

The test solution with concentrations of 100, 50, 25 and 12.5 ppm, each pipette as much as 6 mL was put into a test tube and added 10 shrimp larvae that were 2 days old. Each concentration was repeated twice and compared with the control. Observation I was carried out for 6 hours with an interval of 1 hour. Furthermore, the second observation was carried out at 12, 18 and 24 hours. The number of dead shrimp larvae was counted every 6, 12, 18 and 24 hours [13,14].

2.2. Total Phenolic Test

1 mL of 200 ppm extract was added with 1 mL of Folin-Ciocalteu reagent (50%) in a test tube and then the mixture was vortexed for 3 minutes. After a time interval of 3 minutes, 1 mL of 2% Na2CO3 solution was added. Then the mixture was kept in the dark for 30 minutes. The absorbance of the extract was read by spectrophotometer at 750 nm. The results are expressed as gallic acid equivalents in mg/kg extract [13].

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2.3. GC-MS Test

In identifying the structure of compounds in berenuk fruit with GC-MS, the instruments used were Agilent Technologies 7890 Gas Chromatograph with Auto Sampler and 5975 Mass Selective Detector and Chemstation data system. With an Electron Impact ionization mode of 70 eV. HP Ultra 2 capillary column Length 30 m, ID 0.20 mm, film thickness 0.11 mm. The temperature in the oven is the initial temperature at 80°C, hold for 0 minutes, rise at 3°C/minute to 150°C, hold for 1 minute and lastly rise 20°C/minute to 280°C, hold for 26 minutes [8,15].

The injection port temperature, Ion Source Temperature, Interface Temperature, Quadrupole Temperature are 250°C, 230°C, 280°C, and 140°C, respectively. Helium as carrier gas. 1.2 ml/min constant flow column mode. The injection volume is 5 ml.

2.4. Docking in Silico Testing

Receptor Active Site Determination The potential ligand binding site or pocket (active site) on the 3D structure of the HIV-1 receptor was identified by the Computed Atlas of Surface Topography of proteins (CASTp) web server (http://sts.bioe.uic.edu/castp) [8,16–19]. CASTp uses the latest algorithms and computational chemistry geometric analysis to validate the active site of the receptor.

- Protein Preparation
  The 2F8A receptors (Glutathione peroxidase) and the 3KWZ receptors (Chatepsin K) were downloaded from http://www.rcsb.org, and saved in PDB format (*.pdb). Receptor preparation by removing water, adding H atoms, removing ligands attached to the receptor. Then the protein preparation is stored in pdb (*.Pdb) format

- Ligand Preparation
  The ligands were taken from the GC-MS test results, which then confirmed the compound structure through PubChem http://pubChem.ncbi.nlm.nih.gov, downloaded SDF format file (*.sdf). Through the Chem3D application, the energy of the compound structure is minimized and stored in *.pdb format

- Molecular Docking
  Preparation of ligands and receptors using autodock which is then converted in the form of PDBQT files. The next step is to create a work area through the grid box settings. The grid box is made using the native ligand of the receptor as a reference for the docking process. Only then do the docking process. After docking is complete, the #D image visualization and ligand-amino acid receptor interactions are visualized using an application via the Discovery Studio Visualizer.

3. Results and Discussion

3.1. Toxicity Test (BSLT-Brine Shrimp Lethality Test)

The BSLT test is an acute toxicity test that is carried out to determine the toxic effect after administration of the compound being tested one or more times within a period of 24 hours.

![Probit curve of Sample Extract against log10 concentration.](image)

Plot the graph above and get the equation $y = 0.7416x + 3.8859$ for the n-Hexane extract data and the equation $y = 1.0832x + 2.8653$ for the Toluene extract. Then substitute the value of $y = 5$ to get the value of $x$. In this study, the LC50 values of n-Hexane extract and Toluene extract were 31.79 ppm and 93.49 ppm, respectively. Thus, n-Hexane extract and Toluene extract of berenuk fruit can be said to be toxic.

The extract of n-Hexane of berenuk fruit has the lowest LC50 value of 31.79 ppm, where Meyer (1988) [20] stated that a chemical compound has bioactivity potential if it has a relatively small LC50 or less than 200 ppm, the smaller the LC50 value indicates that the compound The chemical contained in the extract is more toxic or more active.

Phenolic compounds are bioactive secondary metabolites that are widely distributed in various plants, these compounds are divided into sub-groups of phenolic acids, flavonoids, tannins, and stilbenes based on the number of phenolic hydroxyl groups attached and structural elements linking the benzene ring [21]. Phenolic compounds are characterized by one or more aromatic rings (phenolic acid) or more (polyphenols) with a hydroxyl group attached to their structure. It is suspected that its antioxidant capacity is related to this hydroxyl group and phenolic ring [22].
Both extracts have almost the same wavelength, namely; sample was n-hexane extract at 302 nm and toluene extract at 320 nm. This maximum wavelength pattern is a characteristic of the maximum wavelength pattern of the phenolic group at 280 – 320 nm if it is a single peak, while if it is a double peak it will appear at 260 nm and 400 nm [23].

The phenolic content of the bitter gourd was determined by comparing the standard solution of gallic acid and the total phenolic was calculated after the standard curve equation for gallic acid obtained the equation y = 105.54x, r² = 0.9966, where x was the absorbance at 765 nm and y was the total phenolic content. The toluene extract tested had an absorbance of 9.815 or an equivalent phenolic content of 3.96 mg GAE/g sample, while the hexane extract could not detect the presence of phenol.

The results of the partitioning of toluene in the fruit of berenuk contain low phenolic compounds, although Das et., al. 2014, has tested the total phenol content in crude ethanol extract in the leaves of berenuk ranging from 28.07 – 371.23 mg GAE/g of extract, and in the stems of the berenuk plant the total phenolic content ranges from 61.18 - 326.75 mg GAE/g [24]. Although the total phenol content is low, it can also be used to explore new drugs with a limited spectrum of action. This difference is because this study partitioned using n-hexane and toluene in crude ethanol extract, thus allowing a smaller amount of phenolic content.

### 3.2. Identification Structure of Berenuk Compounds with GC-MS

Table 1. GC-MS Result of Berenuk Extract *)

<table>
<thead>
<tr>
<th>No</th>
<th>Sample</th>
<th>RT</th>
<th>Quality</th>
<th>Compound</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.503</td>
<td></td>
<td>95</td>
<td></td>
<td>Benzoic Acid</td>
<td>2.57</td>
</tr>
<tr>
<td>18.963</td>
<td></td>
<td>98</td>
<td></td>
<td>Trans-Cinnamic Acid</td>
<td>8.45</td>
</tr>
<tr>
<td>27.355</td>
<td></td>
<td>95</td>
<td></td>
<td>2-Propenoic acid, 3-phenyl-</td>
<td>1.45</td>
</tr>
<tr>
<td>27.748</td>
<td></td>
<td>22</td>
<td></td>
<td>(E)-4,4-Dimethylpent-2-Enal</td>
<td>2.28</td>
</tr>
<tr>
<td>28.927</td>
<td></td>
<td>98</td>
<td></td>
<td>Docosane</td>
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<tr>
<td>29.430</td>
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<td></td>
<td>Heptadecane</td>
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<tr>
<td>29.755</td>
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<td>95</td>
<td></td>
<td>Tricosane</td>
<td>2.48</td>
</tr>
<tr>
<td>29.892</td>
<td></td>
<td>98</td>
<td></td>
<td>Tetracosane</td>
<td>2.42</td>
</tr>
<tr>
<td>30.313</td>
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<td></td>
<td>Tetracosane</td>
<td>6.99</td>
</tr>
<tr>
<td>30.582</td>
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<td>Tetracosane</td>
<td>5.18</td>
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<td>30.720</td>
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<td>Icosane</td>
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<tr>
<td>30.851</td>
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<td>97</td>
<td></td>
<td>Hexadecane, 1-iodo-</td>
<td>6.54</td>
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<tr>
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<td></td>
<td>Tetracosane</td>
<td>4.77</td>
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<tr>
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<tr>
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<td></td>
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<tr>
<td>31.678</td>
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<td>97</td>
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<td>4.09</td>
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<td>97</td>
<td></td>
<td>Triacontane</td>
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<tr>
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</tr>
<tr>
<td>33.188</td>
<td></td>
<td>96</td>
<td></td>
<td>Hentriacontane</td>
<td>5.85</td>
</tr>
</tbody>
</table>
From the table of GC-MS results above, the compounds contained in the berenuk fruit were taken for docking in silico testing based on the characteristics of phenolic compounds and/or those with relatively large % abundance. The compounds taken from the toluene partition were: Benzoic Acid, Trans-Cinnamic Acid, 2-propenoic Acid, 3 phenyl and 4,4-dimethylpent-4-enal. Meanwhile, the compounds taken from the n-hexane partition were: Trans-Cinnamic acid, 1,2,4-Triazol-4-amine, N-(2-thienylmethyl)- and Benzeneacetic, .alpha.-methoxy-.alpha.–trifluoromethyl-3-methylcyclohexyl ester trans-.

3.3. Molecular Docking In Silico Testing

Molecular docking is an approach method with computer simulation that aims to imitate the interaction of a ligand molecule with the target protein in vitro testing [25]. In other words, molecular anchoring is a process to predict intermolecular interactions. This method can increase the effectiveness in research to find new drug compounds [26,27].

Table 2. Molecular Docking Phenolic Compound Berenuk to Receptor Osteoporosis

<table>
<thead>
<tr>
<th>LIGAN</th>
<th>2F8A Binding Affinity</th>
<th>2F8A RMSD</th>
<th>3KWZ Binding Affinity</th>
<th>3KWZ RMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native ligan</td>
<td>-5.06</td>
<td>0</td>
<td>-7.3</td>
<td>0</td>
</tr>
<tr>
<td>Benzenacetic, .alpha.-methoxy-.alpha.--trifluoromethyl-3-methylcyclohexyl ester trans-</td>
<td>-4.21</td>
<td>2.642</td>
<td>-6</td>
<td>0.792</td>
</tr>
<tr>
<td>Benzoic Acid</td>
<td>-4.49</td>
<td>0.536</td>
<td>-5</td>
<td>0.8</td>
</tr>
<tr>
<td>Cinnamic Acid</td>
<td>-4.81</td>
<td>1.236</td>
<td>-5.4</td>
<td>1.085</td>
</tr>
<tr>
<td>Dimethyl Pent</td>
<td>-3.47</td>
<td>1.08</td>
<td>-3.6</td>
<td>1.438</td>
</tr>
</tbody>
</table>
3.3.1. Binding of Berenuk Extract Ligands to 2F8A Receptors

The 2F8A receptor is a protein modeling enzyme for Glutathione peroxidase (GPx). Glutathione peroxidase (GPx) is the general name of a family of enzymes with peroxidase activity whose main biological role is to protect organisms from oxidative damage. The process of bone remodeling occurs regularly with bone resorption by osteoclasts accompanied by bone formation by osteoblasts [28]. Abnormal conditions cause the function of one of the enzymes to be disturbed so that the bone remodeling process does not run properly and can even cause osteoporosis. While Reactive Oxygen Species (ROS) are involved in bone resorption by contributing directly to osteoclasts that produce superoxide on bone degradation and oxidative stress enhances osteoclast differentiation and function. In addition, osteoblasts produce antioxidants such as glutathione peroxide (GPx) to protect against ROS and osteoblasts also produce transforming growth factor-β, which is involved in bone resorption.

In people with osteoporosis, the rate of bone formation decreases gradually while the rate of bone resorption does not change or increases with advancing age in humans resulting in bone tissue loss. Decreased osteoblast performance can be stimulated as a treatment for osteoporosis. Gpx which is naturally produced by osteoblasts can protect against the damaging effects of ROS.

Osteoporosis therapy drug used as a positive control was minodronate. This compound has a small binding affinity, which is -5.40 kcal/mol. The mechanism of action of Minodronate plays a major role in the inhibition of bone resorption.

Minodronate is the strongest inhibitor of bone resorption among the currently available oral bisphosphonates. Its higher anti-resorption potential for bone mineral and better ability to inhibit osteoclast function [29]. Generally nitrogen-containing bisphosphonates target osteoclast farnesyl pyrophosphate synthase, which is a key enzyme of the mevalonate pathway for inducing cell apoptosis, and blocks protein prenylation, thereby inhibiting bone resorption. [28].

Table 2 shows that benzene acetate, cinnamic acid and propanoic acid are thought to have similar properties to minodronate. This shows that the same bound amino acid residues bind to the 2F8A receptor, namely: THR143, ASP144, LEU147, TRP160, ARG179 and ARG180. The ability of cinnamic acid in the treatment of osteoporosis is explained by [30] that the content of cinnamic acid in cinnamon is able to increase bone turnover by increasing osteocalcin (a molecule exclusively produced by osteoblasts as a marker of bone formation) and increasing osteoblast activity.

On the other hand, [31] explained that cinnamic acid has an anabolic effect on bone mass because it stimulates osteoblastic bone formation and suppresses osteoclastic bone resorption and has a restorative effect on bone loss induced in OVX and diabetic rats. In addition, the cellular and molecular...
mechanisms in which cinnamic acid stimulates osteogenesis and suppresses osteoclastogenesis may be mediated through suppression of NF-κB activation of signaling factors, which play an important role in the regulation of bone homeostasis through inflammatory cytokines.

Figure 5. Surface 2F8A receptor was attached with Ligands Berenuk

Propenoic acid, cinnamic acid and benzene acetate are located in the same position as minodronate. This indicates that the ligand compound of the berenuk extract is able to bind to the receptor with a small binding energy. The hydrogen bond formed refers to the strength of the bond between the ligand and the receptor. Compounds showing a higher negative bond energy contributed to the maximum activity.

3.3.2. Binding of beenuk extract ligands to 3KWZ receptors

The 3KWZ receptor is a modeling receptor protein for Cathepsin K. Cathepsin K (CatK) is a cysteine protease, highly expressed by osteoclasts and highly efficient at degrading type I collagen (a major component of organic bone matrix). The mechanism of inhibition of Cathepsin K in increasing bone mass is by maintaining the alternation of osteoclast (OC) cells with osteoblasts (Ob) during bone remodeling and modeling. CatK inhibitors (CatKi) reduce bone resorption, while significantly increasing the amount of Tartrate-resistant acid phosphatase (TRAP), pre-OC (pOC) on all bone surfaces and multinuclear OC on remodeling surfaces [32]. Tartrate-resistant acid phosphatase (TRAP) is known as an osteoclast marker, sometimes osteoblasts and osteocytes often also appear in the vicinity of bone remodeling sites so that they can also express TRAP.

Figure 6. Binding Affinity Berenuk Ligands with 2KWZ receptor

In the docking of 2KWZ using Raloxifene as a positive control. Some literature mentions the action of raloxifene in relation to Chatepsin K. Whereas Raloxifene in vivo animal ovary trials and clinical trials in postmenopausal women with and without osteoporosis, raloxifene has demonstrated its ability to inhibit accelerated bone resorption, both short and long term, thereby increasing bone density. Bone minerals, preserve bone structure, and increase bone strength. In addition, in a double-blind, placebo-controlled clinical trial in healthy postmenopausal women, raloxifene was shown to significantly decrease bone turnover markers over 24 months characterized by bone content: 15% alkaline phosphate, 30% osteocalcin, and the terminal carbonyl fraction of the collagen type 1 C-telopeptide (CTX) by 40% [33–35].

Table 2 shows that benzene acetate, cinnamic acid and propanoic acid are thought to have similar properties to minodronate. It can be seen that the amino acid residues that are bound to bind to the 2KWZ receptor are: GLY:21, CYS:22, CYS:63, TRP:184, TRP:188 and HIS:162.

Figure 7. Docking Result Receptor 3KWZ with Ligand Compound

Ulcakar and Novinec, 2021 stated that the ability of cinnamic acid and its derivatives to inhibit Cathepsin K is very weak with IC50 values above 1000 M [36]. This is evidenced from the 3D image in Figure 7, it can be seen that the position of cinnamic acid is different from that of Minodronic Acid and Raloxifene. Meanwhile, propenoic acid and benzene acetate compounds have a position close to Raloxifene, this indicates that the compounds that contain 2 ligands of the extract of berenuk are able to bind to the receptor with energy that is almost close to the positive control. And it is suspected that the two compounds of this berenuk extract have the same mechanism of action as the positive control. Although this statement must be proven by in vivo testing.
4. Conclusion

The activity of the compound in *Crescentia cujete* was indicated by the value of the compound’s toxicity. The LC50 values for n-Hexane extract and Toluene extract were 31.79 ppm and 93.49 ppm, respectively. The content of this active compound’s *Crescentia cujete* is a class of phenolic compounds with a phenolic content of 3.96 mg GAEG/g of toluene partition sample, while in hexane extract the presence of phenol could not be detected. Compounds with phenolic groups identified through GC-MS test results are Benzene acetate, cinnamic acid, and propanoic acid. The insilico activity can be seen from the docking results of the Berenik extract compound, the binding affinity values of Benzene acetate, cinnamic acid, and propanoic acid at the 2FA8A receptor are -4.21, -4.81, and -4.72, respectively. While for the 3KWZ receptor, the binding affinity values are -6.0, -5.4, and -5.0, respectively. *Crescentia cujete* fruit extract can be used as an alternative treatment for osteoporosis.

REFERENCES


