Miraxanthin-V, Liriodenin and Chitranone are Hepcidin Antagonist In silico for Iron Deficiency Anemia

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Abstract. Anemia is one of the greatest nutrition problem in the world that is commonly found in children, pregnant women and reproductive women. This disorder is predominantly caused by iron deficiency. Hepcidin, a hepatic hormone, regulates iron metabolism and high serum levels of this hormone are detected in patients with iron deficiency anemia (IDA). Anticalin is a synthetic compound which is able to interact with hepcidin leading to inhibition of ferroportin-hepcidin binding complexes but its therapeutic effects are still under investigation. Indonesia has various herbal plants which are potentially developed to treat some human diseases. Therefore, the purpose of this study was to identify phytochemicals derived from Indonesian plants that is able to inhibit hepcidin-ferroportin interaction. A bioinformatics study with molecular docking method was used in this study. Three-dimensional structures of human hepcidin and anticalin were obtained from the Protein Data Bank (ID: 1M4F and 4QAE respectively). Because their molecular size was big, each molecule was cut into 2 parts of its binding sites. All phytochemicals structures were obtained from HerbalDB and PubChem NCBI database. Truncated anticalin/phytochemicals were molecularly docked with truncated hepcidin by using AutoDock Vina 1.1.2. and their interactions were visualized using PyMol 1.3. Truncated Anticalin had -4.6 and -4.2 kcal/mol binding affinity to truncated human hepcidin. Truncated anticalin 1 was bound to Cys13, Cys14, Arg16, and Ser17 residues in truncated hepcidin 1 while truncated anticalin 2 was at Cys23, and Lys24 residues in truncated hepcidin 2. Miraxanthine-V, Liriodenin and Chitranone had lower binding affinity (-4.8±0.77, -4.7±0.33 and -5.01±0.30 kcal/mol respectively) than that of anticalin and occupied binding sites as same as anticalin did. There are three phytochemicals that potentially become hepcidin antagonists in silico. In vitro assays are required for verification of the antagonist effect of these phytochemicals on iron metabolism.

1. Introduction
Anemia affects a half billion of people in the world and it is generally caused by iron deficiency. In 2011, approximately 29% non-pregnant women who age 15-49 years old and 38% pregnant women suffer anemia [1]. In Indonesia, the prevalence of anemia is 21.7% in baby aged >1 years, 22.7% in children aged 5-12 years, 22.7% in children aged 13-18 years and 37.1% in pregnant women [2]. The Indonesian Ministry of Health has implemented an iron supplementation program to overcome anemia problem especially in pregnant women since 1975. Based on Indonesian Health Profile (2014), the coverage of this program has reached more than 90% for first visit and more than 80% for fourth visit
to health institutions during 2009 - 2012 [3]. However, this program has not been able to reduce the prevalence of anemia in pregnant women [4].

Many studies have reported that some patients with anemia are unresponsive to oral iron supplementation [5, 6]. High levels of serum hepcidin in these patients increase inhibition of iron absorption, which leads to persistent iron deficiency. In recent years, several approaches which are intended to inhibit hepcidin production has been developed to treat this disorder. Modulation of hepcidin production through iron signaling, inflammation, and erythropoiesis pathways is the most common developed approaches whilst antigen therapy is targeted to inhibit hepcidin mRNA. Another approach is inhibition of ferroportin-hepcidin endocytosis or de novo stimulation of ferroportin [7]. Anticalin PRS-080, for instance, is a synthetic molecule which is derived from small extra-cell protein (lipocalin) and able to bind biologically with various endogenous ligands. In addition, anticalin could neutralize human hepcidin hormone in hipoferremia mice [8]. So that anticalin potentially becomes a hepcidin antagonist but efficacy and safety of this drug is further investigated.

Indonesia has approximately 30,000 plant species, which contribute in 80% plant varieties in the world. Around 9.609 plants have therapeutic effects as herbal medicine [9]. Therefore, some phytochemicals from various Indonesian plants may be developed as hepcidin antagonist. Molecular docking is one method of virtual screening which provides valuable information about drug and receptor interaction. So this study aimed to identify Indonesian herbal plants that potentially have hepcidin antagonist by using molecular docking approach.

2. Experimental

All Indonesian herbal plants which were registered in database of HerbalDB were used in this bioinformatics study. Three dimensional structures of hepcidin and anticalin were downloaded from Protein Data Bank (PDB) with accession number 1M4F and 4QAE respectively. While three dimensional structure of phytochemicals were obtained from PubChem NCBI and had to meet Lipinski’s rule of five. PyRx version 0.8 and AutoDock Tools version 1.5.6 were used to molecularly dock between anticalin/phytochemicals and hepcidin. Selected hepcidin antagonists were then visualized using PyMol version 1.3 softwares

Before running molecular docking, water content in hepcidin and anticalin molecules were removed by using AutoDock Tools and added with polar hydrogen molecule. However, both molecules were too big for docking process and these molecules were then cut in the binding site position (grid box) using the same software. Truncated hepcidin had 6 chains (P, Q, R, S, T, and U) and R chain directed the binding site of anticalin. Whereas truncated anticalin had 6 chains (A, B, C, D, E, and F). Residues of Tyr78, Gln174, and Cys175 at A chain, Glu40, Glu44, Val126, and Arg130 at C chain and Lys46 at D chain served as binding sites against truncated hepcidin (Table 1). Grid box I which consisted of 174-176 residues of A chain, 39-41, 43-46 and 128-130 residues of C chain, and 43-46 residues of D chain bound to Cys13, Cys14, Arg16, and Ser17 of truncated hepcidin. Secondly, grid box II which contained 78-79 residues of A chain 125-126 residues of C chain interacted with Cys23 and Lys24 residues of truncated hepcidin.

The position of grid box I was at X center = ±122.794 size = 16, Y center = ±44.184 size = 15, and Z center = ±15.406 size = 12 while grid box II position was at X center = ±132.175 size = 11, Y center = ±36.824 size = 12, and Z center = ±18.427 size = 9. Molecular docking was done at least three times to get root mean square deviation (RMSD) <2 Å. The results of this docking were used as a standard for screening of the phytochemicals. Phytochemicals were then molecularly docked with truncated hepcidin to get binding energy scores. In silico candidates of hepcidin antagonist were selected by comparing their binding energy scores, binding sites and molecular conformation with the standard anticalin.

3. Results and Discussion

Interaction of anticalin and hepcidin was firstly validated to obtain consistent binding energy scores. Truncated anticalin 1 bound to Cys13, Cys14, Arg16, and Ser17 residues of truncated hepcidin 1 in grid
box I with -4.6 kcal/mol binding energy (RMSD 0.0) and the other bound to Cys23 and Lys24 residues of truncated hepcidin 2 in grid box II with -4.2 kcal/mol (Table 1). Cutting process of anticalin and hepcidin molecules should consider their amino acid charges because it will influence their molecular structure and function. Amino acids can be divided into polar (hydrophilic) and non-polar (hydrophobic). The polar amino acid consists of charged amino acids and uncharged amino acids. Polar amino acid with positive charge includes lysine, arginine, and histidine while the ones with negative charge include aspartate and glutamate. Uncharged polar amino acid consists of methionine, glutamine, asparagines, serine, threonine, and cysteine. The non polar amino acid includes glycine, alanine, valine, leucine, isoleucine, and proline. Amino acids in truncated anticalin should have the same charge as the amino acids at its binding site to prevent alteration of atom interaction.
Table 1. Validation of standard anticalin and hepcidin based on binding energy and binding site

| No | Standard Residues | Chain Docking Score | Binding site on Hepsidin | Other residues | Molecule Formula | Lipinski's Rule of Five |
|----|-------------------|---------------------|-------------------------|----------------|------------------|------------------------|
|    |                   |                     | Chain A (Cys14, Arg16)  | Chain C (Cys13, Ser17) | Chain D (Lys24) | C14H26N4O4S | Molecular weight (<500g/mol) | H-Bond Donor (<5) | H-Bond Acceptor (<10) | Compo and lipophility (Log P <5) |
| 1  | Anticalin Standard 1 (Grid Box 1) | Gln174-Xn-Ile176 | A | -4.5 | Cys14, Arg16 | - | - | - | - | - | C14H24N4O6 | 344.363 |
| 2  | Anticalin Standard 1 (Grid Box 1) | Asn39-Xn-Val41 | C | -3.9 | - | - | Cys13, Ser17 | - | - | - | C14H24N4O6 | 344.363 |
| 3  | Anticalin Standard 1 (Grid Box 1) | Gln128-Xn-Arg130 | C | -4.6 | - | - | Cys13 | - | - | - | C15H28N8O5 | 400.433 |
| 4  | Anticalin Standard | Arg43-Xn-Lys46 | C | -3.6 | - | - | Ser17 | - | - | - | C21H38N8O8 | 530.575 |
|   | 1 (Grid Box 1) | 5 | Anticalin Standard 1 (Grid Box 1) | Arg43-Xn-Lys46 | D | -3.7 | - | - | Arg16 | - | C21H38N8O8 | 530.575 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | Anticalin Standard 2 (Grid Box 2) | Tyr78-Xn-Leu79 | A | -4.2 | - | Cys23 | - | - | - | - | C15H22N2O3 | 278.346 |
| 7 | Anticalin Standard 2 (Grid Box 2) | Val125-Xn-Val126 | C | -3.7 | - | - | - | - | Lys24 | - | C10H20N2O2 | 200.278 |
Table 2. Selected phytochemicals as an *in silico* candidate of hepcidin antagonist

| No | Pubchem Code | Compound   | Docking score | Binding site on Hepsidin | Lipinski’s Rule of Five |
|----|--------------|------------|---------------|--------------------------|-------------------------|
|    |              |            |               | Chain A (Cys14, Arg16)   | Chain C (Cys13, Ser17)  | Chain D (Arg16) | Other residues | Molecule Formula | Molecular weight (<500g/mol) | H-Bond Donor (<5) | H-Bond Acceptor (<10) | Compound lipophilicity (Log P<5) |
| 1  | 5281203      | Miraxanthin-V | -4.8          | Arg16 | Cys23 | Arg16 | Cys11 | C15H10O6 | 346.339 | 5 | 8 | 1.1 |
| 2  | 10144        | Liriodenin  | -4.7          | Cys14, Arg16 | - | - | Lys24 | Arg16 | Cys11 | 275.263 | 0 | 4 | 3.4 |
| 3  | 633072       | Chitranone  | -5.1          | Cys14, Arg16 | - | Cys13, Ser17 | - | Arg16 | Cys11 | 374.348 | 2 | 6 | 3.8 |
Identification of potential phytochemicals was started from screening Indonesian herbal plants which have complete molecular structure and small size. There were 517 phytochemicals which met the criteria among 6,776 phytochemicals. After running several times, it revealed that 11 phytochemicals had lower binding energy compared to the standard 1 and 39 phytochemicals had lower binding energy than that of the standard 2. All these compounds interacted with the truncated hepcidin at the same location as the standard did. However, there were only three phytochemicals which had the lowest binding energy (Miraxanthin-V with -4.8, Liriodenin with -4.7 and Chitranone with -5.1 kcal/mol), similar binding sites and conformation, compared with standard (Table 2).

Lipinski criteria which consist of molecular weight, H-bond donor, number of H-bond acceptor and octanol-water partition coefficient (LogP) are usually used to identify new drug candidates. The recommended molecular weight is less than 500 Da. Small molecular weight creates more stable bond and requires less energy to bind. H-bond donor and H-bond acceptor are used to evaluate the ability of a drug to cross cell membrane. As the number of H-bond increases, the cell permeability decreases. LogP or lipophilicity is important to measure the degree of a compound to dissolve in fat. A compound becomes more hydrophilic as the lipophilicity decreases resulting in lower toxicity [10].

We have firstly demonstrated that Miraxanthin-V, Liriodenin and Chitranone potentially become a natural hepcidin antagonist in silico. Miraxanthin-V has five H-bond donors, eight H-bond acceptors and LogP of 1.1. It interacts with hepcidin at Cys$^{11}$, Cys$^{13}$, Arg$^{16}$, Ser$^{17}$, Cys$^{23}$, and Lys$^{24}$. This compound can be found in the stem and root of Mirabilis jalapa plant. It has been used to treat gastrointestinal diseases such as dysentria, diarrhea, muscle pain, abdominal colic and peptic ulcer [11, 12].

Liriodenin does not have H-bond donor but has four H-bond acceptor and logP of 3.4. It interacts with hepcidin at Cys$^{14}$, Arg$^{16}$, Lys$^{24}$ and Thr$^{25}$. Liriodenin interacts with hepcidine at Cys$^{14}$, Arg$^{16}$, Lys$^{24}$ and Thr$^{25}$. This compound can be found in several plants including Annona cherimolia, Annona reticulata, Nelumbo nucifera and Polyalthia Longifolia. Liriodenin was reported to inhibit proliferation of ovarian cancer cell and have various biological activities as antimicrobial, antifungal, anti-tumor, anti-platelet, anti-aritmia, sedative and anti-depressant effect [13, 14, 15].

Chitranone has two H-bond donors, six H-bond acceptors and LogP of 3.8. Chitranone interacts with hepcidine at Cys$^{11}$, Cys$^{13}$, Cys$^{14}$, Arg$^{16}$, and Ser$^{17}$. This compound can be found in the leaves and root of Plumbago Zeylanica [16]. Chitranone has several therapeutic properties such anti-diabetes, anti-cholesterol, anti-inflammation, anti-cancer, anti-microbial, anti-malaria and anti-fertility property [17].
| Chain | Standard | Miraxanthine-V | Liciodenine | Chitraneone |
|-------|----------|----------------|-------------|-------------|
| A CHAIN (78-79) | ![Image](image1.png) | ![Image](image2.png) |  |  |
| C CHAIN (39-41) | ![Image](image3.png) | ![Image](image4.png) |  |  |
| C CHAIN (43-46) | ![Image](image5.png) | ![Image](image6.png) |  |  |
| C CHAIN (125-126) | ![Image](image7.png) | ![Image](image8.png) |  |  |
| C CHAIN (128-130) | ![Image](image9.png) | ![Image](image10.png) |  |  |
| D CHAIN (43-46) | ![Image](image11.png) | ![Image](image12.png) |  |  |

**Figure 1.** Visualization of anticalin/phytochemicals and hepcidin binding complexes using PyMol. Green: Carbon (C), Red: Oxygen (O), White: Hydrogen (H), Blue: Nitrogen (N), Yellow: Sulfur (S), dashes-line: atomic interactions, white boxes: amino acid residues.

We realized that this study has limitations. One of them is the fact that anticalin molecule that we used was a modified molecule obtained by cutting anticalin-hepcidin molecule complex. In addition, the standard molecule anticalin and target protein, hepcidin, are large protein. AutoDock Vina program can only perform protein-ligand docking. Thus, we modified anticalin and hepcidin molecule by cutting several residues. It is likely that this modification influence the docking result.
4. Conclusion
Miraxanthin-V, Liriodenin, and Chitranone potentially become hepcidin antagonist *in silico* which have higher binding affinity and molecular similarity, compared with the standard anticalin. In vitro assays should be performed to evaluate the antagonist effects of these phytochemicals on iron regulation.

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