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Research Article

The Potential of Bioactive Compounds in Beets (*Beta vulgaris* L.) to Interact with the HFE Protein in Anemia: an in Silico Study

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Abstract

Anemia was a global health problem associated with low hemoglobin levels and impaired iron homeostasis. The Homeostatic Iron Regulator (HFE) protein played a role in regulating iron metabolism through the hepcidin pathway, making it a relevant target for computational studies. This study aimed to evaluate the potential interactions of secondary metabolites from beetroot (*Beta vulgaris* L.)—namely betacyanin, catechin, and gallic acid—with the HFE protein using an *in silico* molecular docking approach. The HFE protein structure (PDB ID: 1DE4) was prepared using PyMol, while the docking process was performed using PyRx 0.8 based on AutoDock Vina. N-acetyl-D-glucosamine (NAG) was used as the control ligand to compare binding affinity and interaction sites. The docking results showed that all three test compounds exhibited more negative binding affinities than NAG (−3.1 kcal/mol), in the following order: betacyanin (−4.4 kcal/mol), catechin (−4.0 kcal/mol), and gallic acid (−3.6 kcal/mol). All three compounds also exhibited interactions with the GLU383 residue, similar to the control ligand. These findings suggested that the bioactive compounds in beetroot had the potential to interact spontaneously and stably with the HFE protein at the computational level, with betacyanin emerging as the most promising candidate. However, these findings remained predictive and required further validation through *in vitro* and *in vivo* studies before they could be developed as potential agents to support anemia therapy.

Keywords: anemia; *Beta vulgaris L.*; betacyanin; druglikeness; HFE; in silico

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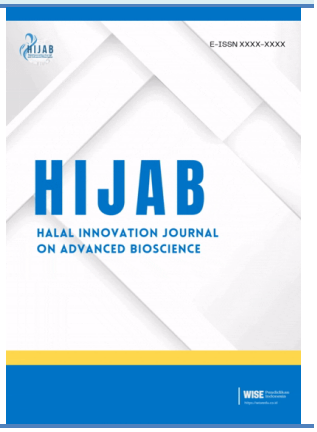


INTRODUCTION

Anemia remains a major global public health problem with a high prevalence, particularly in Indonesia. It is characterized by a reduction in the number of red blood cells (erythrocytes) or hemoglobin (Hb) concentration below the normal threshold (<13 g/dL in men and <12 g/dL in women), resulting in impaired oxygen transport to body tissues [1]. According to Shaka and Wondimagegne, iron deficiency and genetic factors are the most common causes of anemia. Rather than being a disease itself, anemia is often an early clinical manifestation of various underlying disorders. Therefore, in addition to confirming the diagnosis of anemia, identifying its underlying cause is essential for appropriate clinical management. Preventive strategies include the consumption of iron-rich foods and dietary antioxidants that support erythropoiesis and iron metabolism.

The Homeostatic Iron Regulator (HFE) protein does not directly stimulate hemoglobin synthesis; however, it plays a critical role in maintaining systemic iron homeostasis through the hepcidin signaling pathway and its interaction with the transferrin receptor. HFE is a membrane glycoprotein structurally homologous to major histocompatibility complex (MHC) class I molecules and forms a complex with β2-microglobulin (β2M). The HFE protein binds to transferrin receptor 1 (TFRC) via its extracellular α1–α2 domains, an interaction that is essential for regulating hepatic hepcidin expression and maintaining iron homeostasis [2]. Dysregulation of HFE function may alter hepcidin expression, ferroportin activity, intestinal iron absorption, and iron availability for erythropoiesis, thereby contributing to the development of anemia [3].

One natural product reported to improve hemoglobin levels is beetroot (*Beta vulgaris* L.). Beetroot is a nutrient-rich root vegetable distinguished by its characteristic reddish-purple color and recognized for its health-promoting properties. It contains folic acid, iron, and vitamin C, all of which contribute to hemoglobin synthesis and may help prevent or alleviate anemia. A previous study involving 17 adolescent girls with anemia demonstrated that daily consumption of 250 mL of beetroot juice for seven consecutive days increased the mean hemoglobin concentration from 10.682 g/dL to 11.088 g/dL [4]. Statistical analysis using a paired *t*-test further demonstrated that beetroot juice significantly increased hemoglobin levels in pregnant women with anemia. Beetroot also contains iron (Fe), an essential component of the heme group responsible for hemoglobin formation [5]. In addition to vitamins and minerals, beetroot contains several bioactive secondary metabolites, including betacyanin (37 µg), catechin (184.50 µg), and gallic acid (137.23 µg).

Gheith and El-Mahmoudy (2018) reported that beet leaf extract significantly restored red blood cell count, white blood cell count, hemoglobin concentration, and hematocrit values in a dose- and time-dependent manner in an *in vivo* study [6]. The anti-anemic activity of beetroot extract has been attributed primarily to its flavonoid and phenolic constituents. Similarly, Elaby and Ali identified betacyanins, betaxanthins, flavonoids, polyphenols, and vitamins in beet leaves through phytochemical and chromatographic analyses [7]. Furthermore, Abdo et al. reported that the principal anti-anemic constituents of beetroot include betacyanins (particularly betanin), which function as potent antioxidants that protect erythrocytes against oxidative damage; iron, which is essential for hemoglobin synthesis; vitamin C, which enhances iron absorption; and folate, magnesium, and nitrate, which support hematopoiesis and improve hemoglobin concentration, hematocrit, and erythrocyte count [8]. These studies predominantly employed *in vivo* and *in vitro*experimental approaches.

Previous investigations on beetroot have primarily focused on *in vivo* and *in vitro* approaches to evaluate its effects on hematological parameters. However, these studies have not specifically identified the protein targets involved in iron homeostasis or elucidated the molecular interactions between beetroot bioactive compounds and proteins associated with iron regulation. Therefore, an *in silico* molecular docking approach is needed as an initial step to predict binding affinity, interaction

sites, and the stability of interactions between betacyanin, catechin, and gallic acid and the HFE protein [9].

Based on this research gap, the present study aimed to evaluate the binding potential of three major secondary metabolites from beetroot—betacyanin, catechin, and gallic acid—toward the HFE protein, a key regulator of iron homeostasis implicated in anemia. Specifically, this study analyzed binding affinity values, interacting amino acid residues, similarity of binding sites with the control ligand, and drug-likeness characteristics based on Lipinski's Rule of Five. The findings provide preliminary computational evidence supporting the potential of beetroot-derived bioactive compounds as candidate agents for anemia management and establish a scientific basis for subsequent experimental validation.

METHODS

This study was conducted using an *in silico* approach employing a molecular docking technique. The workflow consisted of target gene identification, active compound selection, ligand preparation, protein preparation, grid box determination, molecular docking simulation, and visualization of ligand–protein interactions. The test ligands included catechin, gallic acid, and betacyanin, while *N*-acetyl-D-glucosamine (NAG) was used as the control ligand to compare binding patterns and interaction sites with the HFE protein.

Target Gene Identification

a. Identification of Anemia-Associated Genes
   Genes associated with anemia were identified using the PheWAS Catalog by searching with the keyword "iron deficiency." The identified genes were subsequently screened for missense variants using the dbSNP database.
b. Functional Annotation and Target Prioritization
   Genes containing missense variants were further prioritized through functional annotation based on six biological criteria: missense mutation, Biological Process (BP), Cellular Component (CC), Molecular Function (MF), biological pathways, and gene expression (cis-eQTL) in whole blood cells. Each annotation was assigned a binary score, with 1 indicating the presence of a functional annotation and 0 indicating its absence. Genes with a total score greater than 2 were selected as candidate target proteins because they fulfilled more than two functional criteria, suggesting a stronger biological association with anemia and iron metabolism. This threshold served as an initial filtering criterion to prioritize the most relevant targets for subsequent molecular docking analysis.

Selection of Active Compounds

Based on the study by Abdo, El-Sohaimy, et al. [8], betacyanin, catechin, and gallic acid were identified as the predominant bioactive constituents of beetroot (Beta vulgaris L.). Therefore, these three compounds were selected as the test ligands for molecular docking analysis.

Ligand Preparation

The chemical structures of catechin, gallic acid, and betacyanin were obtained in Structure Data File (.sdf) format. The ligand structures were opened in PyMOL to remove water molecules and other unnecessary components before being saved again in .sdf format. N-acetyl-D-glucosamine (NAG), the co-crystallized ligand of the target protein, was used as the reference ligand to compare binding locations and interaction characteristics. In this study, NAG was not considered evidence of biological

activity but was employed as a computational reference because its presence in the crystal structure facilitated the identification of relevant amino acid residues surrounding the binding pocket.

Protein Preparation

The target protein structure was selected based on several quality criteria, including a crystallographic resolution of <3 Å, a positive G-Factor value, and the absence of missing residues that could interfere with the interpretation of ligand–protein interactions. The crystal structure of the HFE protein (PDB ID: 1DE4) was retrieved from the Protein Data Bank (PDB) in .pdb format. Protein preparation was performed using PyMOL by removing water molecules and other non-essential components before molecular docking.

Molecular Docking

The prepared HFE protein structure was imported into PyRx version 0.8, which utilizes the AutoDock Vina 1.1.2 docking engine. After importing the protein and ligand structures, the grid box was centered on the binding region of the reference ligand to ensure that docking simulations were performed within the biologically relevant binding pocket. The docking results were exported in .pdb format and subsequently analyzed using Discovery Studio to identify interaction types, interacting amino acid residues, and the ligand–protein interaction patterns.

RESULT AND DISCUSSIONS

Target Protein Preparation

Target protein preparation began with the identification of genes associated with anemia using the PheWAS Catalog. An initial search retrieved approximately 212,000 genes, which were subsequently filtered using the keyword "iron deficiency," resulting in 167 candidate genes. These genes were then screened for missense variants using the dbSNP database, yielding four genes harboring missense mutations (Table 1).

The final target gene was selected through a functional annotation scoring approach. The four candidate genes were analyzed using the STRING database (https://string-db.org), and each gene was evaluated based on six functional criteria: missense mutation, Biological Process (BP), Cellular Component (CC), Molecular Function (MF), biological pathways, and gene expression (cis-eQTL) in whole blood. Each criterion was assigned a binary score, where 1indicated the presence of functional evidence and 0 indicated its absence. Genes with a total score greater than 2 were considered biologically relevant and prioritized as potential molecular targets. Based on this scoring system, the Homeostatic Iron Regulator (HFE) gene achieved the highest score (4) and was therefore selected as the primary target protein (Table 1).

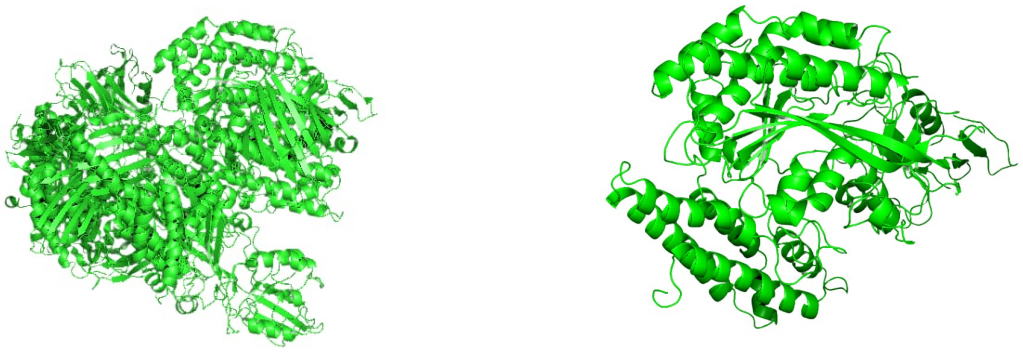
The HFE protein was selected because of its central role in regulating systemic iron homeostasis, a biological process closely associated with iron-deficiency anemia. The HFE protein consists of 343 amino acid residues and contains extracellular α1 and α2 domains responsible for transferrin receptor binding, an immunoglobulin-like α3 domain, a transmembrane region, and a short cytoplasmic tail. Rather than directly stimulating hemoglobin synthesis, HFE regulates iron metabolism through the hepcidin signaling pathway, thereby influencing iron availability for erythropoiesis and hemoglobin production. Consequently, HFE represents a biologically relevant molecular target for investigating compounds with potential anti-anemic activity.

The three-dimensional structure of the HFE protein was retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (https://www.rcsb.org/), which provides experimentally determined protein structures in Protein Data Bank (PDB) format. The crystal structure with PDB ID: 1DE4 was selected because it fulfilled the quality criteria established in

this study, including a crystallographic resolution of 2.60 Å, a positive G-Factor value (+0.22), and the absence of missing residues that could interfere with molecular docking analysis.

Table 1. Distribution of Gene Annotation Based on Functional Categories

Gene	Missense	Biological Process	Cellular Component	Molecular Function	Biological Pathways	Gene Expression (Cis-eQTL)	Total
HFE	1	0	1	0	1	1	4
SH2B3	1	0	0	0	0	1	2
SLC30A8	1	0	0	0	0	0	1
TMPRSS6	1	0	0	0	0	1	2
Total	4	0	1	0	1	3	9



a. b.

Figure 1. a. HFE protein (1DE4) before purification, b. HFE protein (1DE4) after purification

Molecular Docking

Molecular docking was performed using the Pyrx program. Pyrx combines the OpenBabel library for ligand preparation and AutoDock VINA for running the molecular docking simulations. The ligands from secondary metabolites of beets used in the testing were catechin, gallic acid, and betacyanin, while the reference ligand was NAG (N-acetyl-β-D-glucosaminidase). A compound capable of binding to HFE with interaction characteristics similar to those of NAG has the potential to exhibit the same activity as its reference ligand. The test parameters analyzed include the binding affinity between the compound and the protein—measured by free energy—as well as the binding site between the test ligand and the target protein. The more negative the binding affinity value, the stronger the binding interaction between the receptor and the ligand, and thus the greater the ligand's inhibitory effect on the target macromolecule [10]. The coordinate results are summarized in Table 2.

Table 2. Determination of Coordinate Points (Grid Boxes)

	Coordinate (*Angstrom*)		
	X	**Y**	**Z**
Center	49.7936	70.2208	134.6585
Dimension	14.9064	10.7229	9.0136

Based on the docking analysis, the interaction between NAG and the HFE protein showed a binding affinity of -3.1 kcal/mol. A negative energy value indicates that the ligand-protein interaction can occur spontaneously under computational conditions. Betacyanin exhibited the most negative binding affinity value, namely -4.4 kcal/mol, followed by catechin at -4.0 kcal/mol and gallic acid at -3.6 kcal/mol. Values more negative than that of NAG indicate that the three test compounds have the potential for stronger binding affinity toward HFE compared to the control ligand, although this interpretation remains predictive and cannot yet be equated with proven biological activity.

Of the three test compounds, betacyanin showed the greatest potential for interaction because it had the most negative binding affinity value. These findings suggest that betacyanin is a prime candidate for further analysis, particularly since its interaction is determined not only by the binding energy value but also by the type of bond and the compatibility of amino acid residues at the binding site.

Table 3. Binding Energy of Ligands with HFE

Target Protein	Test Compound	(Kcal/Mol)
HFE (1DE4)	*NAG*	-3.1
	Catechin	-4.0
	Gallic acid	-3.6
	Betacyanin	-4.4

Table 4. Types of Interactions and Locations of Interactions with HFE

Test Compound	Type of Interaction	Interaction Site
NAG	Hydrogen Bond	*ASN317*
	Hydrogen Bond	*GLU383*
Catechin	Hydrogen Bond	*GLU383*
Gallic Acid	Hydrogen Bond	*GLU383*
	Hydrophobic Interaction	*PHE187*
Betacyanin	Electrostatic	*GLU383*
	Hydrogen Bond	*LYS385*
	Hydrophobic Interaction	*PHE187*

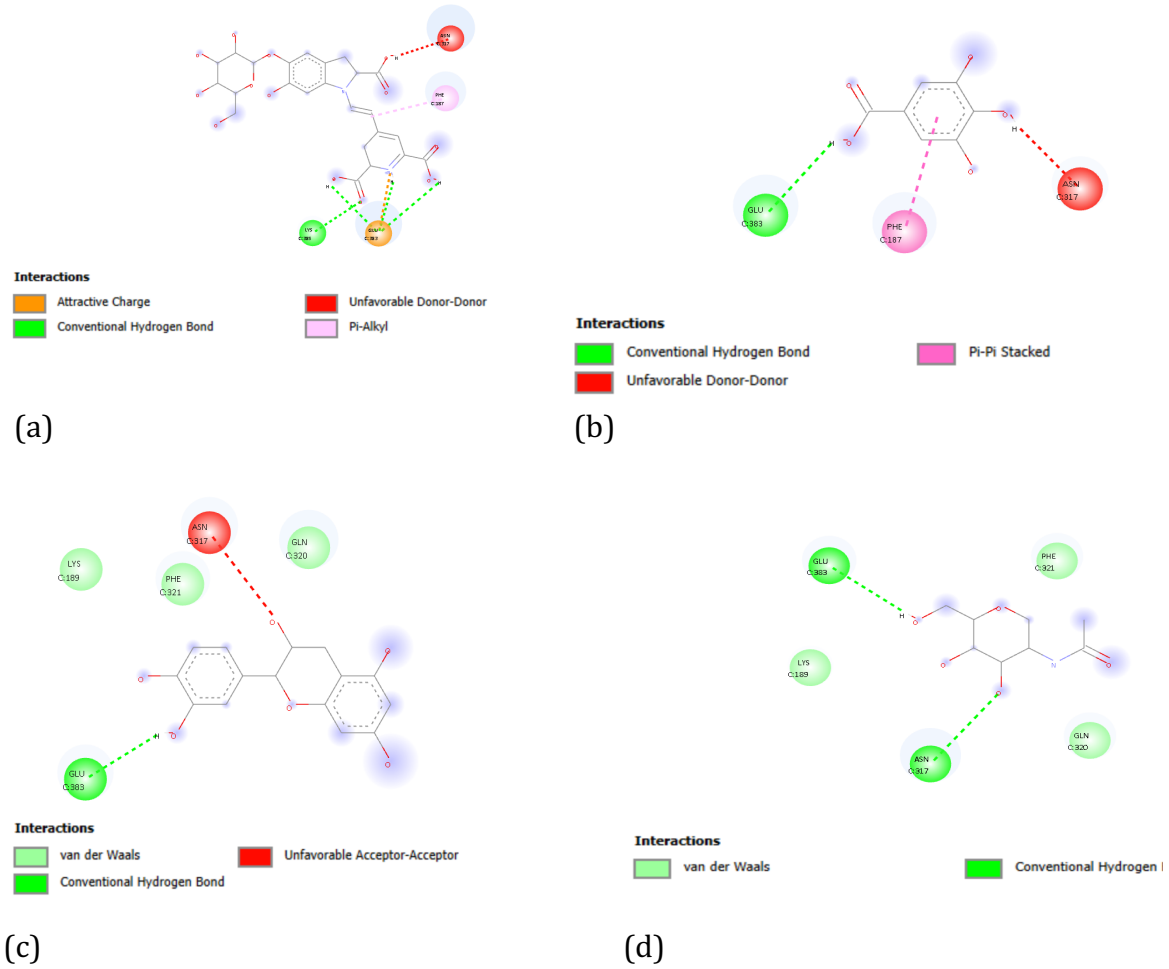


Figure 2. Interaction of the Sample with HFE: a. *Betacyanin* b. *Gallic acid,* c. *Catechin* d. NAG

Interactions between compounds and proteins can occur due to chemical bonds between the two molecules, such as hydrogen bonds, hydrophobic interactions, and electrostatic interactions. Hydrogen bonds at specific sites can help stabilize the ligand-protein complex and contribute to the proper orientation of the ligand within the binding pocket [11]. If a compound interacts at a location relatively similar to that of a reference ligand, it can be hypothesized that the compound has a similar interaction pattern at the computational level. Thus, in this study, the similarity of interaction sites with NAG was used as an initial indicator to assess the potential for interaction of the test compounds with the HFE protein, rather than as direct evidence of biological efficacy.

Based on the study results, the control ligand NAG and the test compounds betacyanin, catechin, and gallic acid all showed interaction with the GLU383 residue. The visualization results in Figure 2 show that betacyanin forms an electrostatic interaction with GLU383, a hydrogen bond with LYS385, and a hydrophobic interaction with PHE187. Gallic acid forms a hydrogen bond with GLU383 and a hydrophobic interaction with PHE187, while Catechin forms a hydrogen bond with GLU383. The involvement of the GLU383 residue is significant because this residue is also found in the interaction between NAG and HFE, thus supporting the hypothesis that the test compounds have a relevant interaction orientation within the binding pocket.

Druglikeness

Druglikeness was evaluated using Lipinski's Rule of Five as an initial indicator of a compound's physicochemical suitability. Lipinski's Rule states that ideal oral drug candidates generally have fewer than 10 hydrogen bond acceptors, fewer than 5 hydrogen bond donors, a ClogP value of less than 5, and a molecular weight of less than 500 Da. Based on Table 5, betacyanin has 8 hydrogen bond donors, 14 hydrogen bond acceptors, a molecular weight of 550.470, a ClogP of -1.50, and a polar surface area of 247.11 $Å^2$. These values indicate that betacyanin violates several of Lipinski's criteria, particularly regarding molecular weight and the number of hydrogen bond acceptors and donors. Therefore, betacyanin cannot be immediately concluded to be an ideal candidate for an oral drug, but it can still be considered a candidate for a bioactive compound that requires optimization, further pharmacokinetic studies, and experimental validation. The interpretation regarding the possibility of intravenous administration should be understood as a preliminary hypothesis that has not yet been pharmacologically proven.

Table 1. Lipinski Rule's of Five Druglikeness

SMILE	Genes	Hydrogen Bond Donors	Hydrogen Bond Acceptors	Molecular Weight	ClogP	Polar Surface Area
C1[C@H(N=C(C=C1/C=C/N2[C@@H(CC3=CC(=C(C=C32)O)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)CO)O)O)O)C(=O)O)C(=O)O)C(=O)O	Betacyanin	8	14	550.470	-1.50	247.11 A^2
C1[C@@H]([C@H](OC2=CC(=CC(=C21)O)O)C3=CC(=C(C=C3)O)O)O	Catechin	5	6	290.27SS	0.83	110.38 A^2
C1=C(C=C(C(=C10)O)O)C(=O)O	Gallic acid	4	5	170.12	0.21	97.99 A^2

Based on the docking results and druglikeness evaluation, secondary metabolites from beets show potential for interaction with the HFE protein, with betacyanin emerging as the most promising candidate based on binding affinity values and residue interaction patterns. However, the conclusions that can be drawn from this study are still limited to computational potential. This study has not yet demonstrated a direct effect on hemoglobin levels, has not tested the stability of the complex through molecular dynamics simulations, and has not evaluated bioactivity using cellular or animal models. Therefore, the results of this study should be used as a basis for selecting candidate compounds for in vitro and in vivo testing in subsequent stages.

CONCLUSION

Betacyanin, catechin, and gallic acid can interact with the HFE protein. The types of bonds involved in the interactions between betacyanin, catechin, and gallic acid with the HFE protein are hydrogen bonds, hydrophobic bonds, and electrostatic bonds; hydrophobic interactions are formed between betacyanin and gallic acid. Of these three compounds, betacyanin exhibits the greatest potential for HFE inhibition. Furthermore, betacyanin's druglikeness suggests that it has the potential to be developed into a drug that can be administered intravenously, particularly.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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