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Original Research Article

Antianaemic Potential of *Flavonoids* from *Ajwa Date Fruits*: An *In Silico* Study

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ABSTRACT

Iron deficiency anemia (IDA) is a hematologic disease that can occur in all age groups. Globally, it is estimated that 29-43% of women of child bearing age (15 – 49 years) are affected by anaemia. A majority of those affected are pregnant women and adolescent girls. Erythropoietin (EPO) is currently being used as one of the biomarkers of anemia. This study aimed to predict the antianaemic potential of *flavonoids* extracted from *Ajwa Date* Fruit through molecular docking.
The *in silico* study was done using Autodock Vina software with VegaZZ, PyMOL, and BIOVIA Discovery Studio programs to create visual profiles of EPO native ligand together with six test compounds; *Flavocommelin, Complanatuside, Isoschaftoside, Kaempferol-3-O-gentiobioside, Kaempferol-3-O-rutinuside, and Spinosin.* Pharmacokinetic predictions were done using the pkCSM approach. Post-docking analyses such as binding affinities and pharmacokinetic predictions showed that the *flavonoid* compounds have high binding free energy, similar to standard therapeutic agent (iron, Fe), and exhibited excellent pharmacokinetic profile. The *flavonoid complanatuside* (4-(2- Hydroxyethyl -1- piperazine ethanesulfonic acid) had the best binding affinity with docking score of -5.01 kcal/mol (371.11%), which was comparable to the docking score of the positive control ligand (Fe) (-4.37 kcal/mol). The root mean square deviation (RMSD) values for EPO were 0.710 Å, 0.300 Å, and 2.007 Å. Therefore, *flavonoids* from *Ajwa date fruits* especially *complanatuside* have the potential to be used as natural compounds for the treatment of iron deficiency anaemia.

Keywords: Antianaemic *Flavonoids*, Erythropoietin, Molecular docking.

Introduction

Iron deficiency is a common cause of anemia. Iron deficiency anemia (IDA) is characterized by low hemoglobin levels in the red blood cells, which reduces the ability of red blood cells to transport oxygen to other tissues.[1] Anemia is a common medical problem worldwide, and the World Health Organization(WHO) defines anemia as hemoglobin levels less than 12 g/dL for women and less than 13 g/dL for men. IDA is a hematologic disease in all age groups, especially women.[2] Anemia can be found in infants, children, preschoolers, menstruating young women, women in the second/third trimester of pregnancy and postpartum women.[3] The global prevalence of anemia is 36.5% among pregnant women, 29.6% in non-pregnant women, and 39.8% in children aged 6–59 months.[4,5] Indonesia has the highest prevalence of anemia among pregnant women (48.9%), followed by toddlers (38.5%), and adolescent girls (32%).[6]
Ajwa date is a plant that has been found to have antianaemic properties. Active compounds contained in the plant have been found to increase haemoglobin (Hb) concentration in the red blood cells.

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Thus, *Ajwa date* has been used as a high-energy food for non-pharmacological alternative therapy for IDA. *Ajwa dates* contain Iron, carbohydrates, proteins, magnesium, zinc, folic acid, fats and fiber, minerals, vitamins, and antioxidants. It also contain many *flavonoid compounds*, *polyphenolic compounds*, *phenolics*, *carotenoids*, and vitamins as antioxidants that maintain heme iron in the ferrous state.[7-9]
Erythropoietin (EPO) is a hormone that stimulates erythrocyte formation in the bone marrow (erythropoiesis). EPO is produced in the proximal interstitial tubular cells of the kidney, it is currently being developed as a biomarker for anaemia. The sensitivity of erythroblasts erythropoietin can be modulated by transferrin receptor 2 (TFR2). Erythroblasts and erythrocytes donate iron through Ferroportin-mediated iron export (FPN).[10] The hormone erythropoietin (EPO)controls the proliferation of erythroid progenitors, the initial phase of terminal erythropoiesis, while the need for iron increases in the late stage of differentiation of proerythroblasts into reticulocytes, for the synthesis of heme and its incorporation into haemoglobin.[11]
Designing and developing new compounds as antianaemic agents and testing them *in silico* through molecular docking approach have advantages over *in vivo* and *in vitro* approaches. In addition to predicting mechanisms of action, biochemoinformatics studies can predict pharmacokinetic profiles and toxicities. The pharmacokinetic and toxicity profiles are important because they are used to determine the presence of active substances in plants and their possible side effects.[12,13] How the docking input structure is configured is just as crucial as the docking process itself, and interpreting the results of stochastic search methods can sometimes be challenging.[14] Therefore, the present study aim to determine the potential of *flavonoids* from *Ajwa*

Ajwa date fruits as antianaemic therapies through *in silico* approach using EPO as target protein.

Materials and Methods

Software and applications

The instruments, software, and applications used in this study include Asus i5 laptop, and AutoDock4 Tools (v1.5.7). The structures of the test ligands (active compounds of Ajwa date fruits) were drawn using the Vega ZZ application and downloaded in 3D format from the Pubchem website (htpps://pubchem.ncbi.nlm.nih.gov/). EPO protein target structure with PDB ID: 6MOE, PDB DOI: https://doi.org/10.2210/pdb6MOE/pdb was downloaded from www.rscb.org. The docked ligands with EPO proteins were visualized using the BIOVIA Discovery Studio. SwissADME programs were accessed from the website (http://www.swissadme.ch.Specialist). Toxicity prediction was done using the pkCSM web server (http://structural.bioc.cam.ac.uk/pkcsm).

Protein and ligands preparations

The application Biovia DS Visualizer was used to make EPO protein and ligands. It involves the extraction of proteins from native ligand and amino acid residues and then storing all files. The ligands used in the study were *flavonoids* previously isolated from the ultrasound assisted aqueous extract of *Ajwa date fruits*. The *flavonoids* are *Flavocommelin, Complanatuside, Isoschaftoside, Kaempferol-3-O-gentiobioside, Kaempferol-3-O-rutinuside, and Spinosin.* Ligand preparation was carried out by optimizing the 3-dimensionalstructure of the *flavonoids* using the Vega ZZ application. Each compound obtained from the Pubchem (http://pubchem.ncbi.nlm.nih.gov) database was then stored in PDB format and converted into GDPQ using the Autodock Tool. The steps included removing unnecessary parts, adding hydrogen, adding gastaiger charge, and placing the gird box in the center of the native ligand in the active site of the receptor containing amino acid residues.[15,16]

Docking parameter validation

AutoDock software (v1.5.7) was used to validate the docking method in this study.The validation process involves redocking the native ligand of the EPO protein. The results of this process include grid parameters and root mean square deviation (RMSD). RMSD is a measure of the mean distance value that correlates with the size of the docking zone. The goal is to achieve an RMSD value of less than 2 Å. The docking method is valid if the RMSD is less than 2 Å. If the RMSD exceeds 2 Å, the method used is invalid. Next, the Grid submenu and the Grid panel were used to set the protein and ligand docking regions.[17]

Molecular docking

Autodock Vina software (v1.5.7) was used to perform EPO molecular docking. The grid box parameters were specified, and the docking and RMSD scores were obtained from the docking process by typing vina.exe -config conf.txt -log log.txt at the Windows command prompt. The EPO protein pdbqt and the *flavonoid* compound pdbq were written to a new document named conf.txt. On the other hand, the ligand was represented as ligand.pdbqt, with centers x, y, z and sizes x, y, z. Values specified in the grid fields were used to get the RMSD value of the docking process, by using the command "vina.exe -config conf.txt -log log.txt" at the Windows command prompt or by typing "cmd" in the folder address and pressing Enter.

This code will display multiple conformations, docking scores, and the RMSD values for each conformation. The success parameter observed during docking method validation is the root mean square deviation (RMSD) value. The docking method was validated using AutoDock software (v1.5.7) (http://autodock.scripps.edu/, Redocking of Native Ligands of EPO Proteins) (GDP DOI: https://doi.org/10.2210/pdb6MOE/pdb). The result of this process was obtainedin the form of grid parameters and root mean square deviation (RMSD). The RMSD value is associated with the size of the docking zone.

Adjustments were done to achieve RMSD values ≤ 2 Å, which is considered good enough for docking.[18,19]

Molecular docking data visualization

The Biovia DS Visualizer 2021 (v21.1) app was used for molecular docking visualization. The visualization process aims to identify the spatial arrangement and visual representation of the bonds between proteins and ligands inthree dimensions. The visualization results were then analyzed to assess compound interactions and determine the position and visual depiction of protein binding of each ligand tested. Analysis of the molecular interactions formed was used to identify the conformation of *flavonoid* compounds and their binding interactions (hydrogen and non-hydrogen bonding) on the protein active sites. The data were stored in the pdbqt file format.[20]

Pharmacokinetic analysis

The pkCSM web server was used to predict the pharmacokinetic profiles of the test ligands. The parameters predicted include absorption, distribution, metabolism, and excretion (ADME) using the SwissADME program (https://www.swissadme.ch), the physicochemical properties using the Lipinski rule of five, as well as the oral bioavailability.[21-23]

Toxicity analysis

Toxicity predictions were made using the Toxtree app. The following toxicological parameters were assessed: Ames toxicity, the maximum tolerable dose in humans, inhibition of hERG-I, inhibition of hERG-II, acute oral toxicity in *rats* (LD_{50}), chronic oral toxicity in mice, hepatotoxicity, skin sensitization, and toxicity to *Tetrahymena pyriformis*.[24,25]

Results and Discussion

In silico study was conducted to determine the binding interaction between *flavonoid* compounds from *Ajwa date* fruits and EPO receptors. The molecular docking parameters were validated by redocking the native ligand (ACI) to the active site of the EPO receptor. The selected molecular parameters were subjected to 50 runs with grid box points of 40 x 40 x 40 and grid box coordinates x, y, z of 14.514, 18.915, and 17.468, respectively.

Molecular docking is a useful tool in drug design, its predict the binding affinity of test ligands to functional target proteins. Other applications of the *in silico* approach include pharmacokinetic prediction using pkCSM models. The main result of the molecular docking procedure is binding affinity, commonly referred to as the docking score. The binding affinities of the test ligands *(flavonoid* compounds), native ligands and positive control ligand (Fe) are presented in Table 1. The native ligand gave a docking score of -1.35 kcal/mol (100%), while the docking score (kcal/mol) of the test ligands are as follows; *Flavocommeli*n -3.20 (237.03%), *Complanatuside* -5.01 (371.11%), *Isoschaftoside* -3.15 (233.30%), *Kaempferol-3-gentiobioside* -1.25 (9.26%), *Kaempferol-3-O-rutinuside* -4.01 (297.03%), and *Spinosin* -2.69 (199.25%). The most excellent binding affinity was obtained for the test ligand *Complanatuside* (4-(2-Hydroxyethyl-1-piperazine ethanesulfonic acid) with binding affinity of -5.01 kcal/mol (371.11%). The compound *complanatuside* also exhibited a better binding affinity for EPO than Fe (-4.37 kcal/mol). The root mean square deviation (RMSD) was calculated for the docked ligand with the lowest binding energy during the docking process.

The test is valid if the RMSD value obtained is ≤ 2 Å. During this study, the RMSD value for EPO protein was 0.710 Å, 0.300 Å, and 2.007 Å, with an average RMSD value of 0.505 ± 0.289 Å. Smaller RMSD value indicates slightly different structural conformation during docking.

Table 2 shows the 3D visualization of the interaction between the test ligands and receptor. The ligands interacted with the amino acid residues of EPO via hydrogen bonding and other non-hydrogen bond interactions. The best interaction was obtained for the compound *complanatuside* with hydrogen and non-hydrogen bonds interaction with the amino acids Ile36, Trp37, and Ile69 of EPO protein.

On the basis of the binding affinity and RMSD values obtained, the *flavonoid* compound *complanatuside* has potential antianemic effects that could be comparable to standard therapy (Fe). The SwissADME program was used to predict the pharmacokinetic parameter of absorption, distribution, metabolism and excretion of the *flavonoid*

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compounds, in addition, physicochemical properties as a measure of the druglikeness of the test ligands were also predicted using the pkCSM strategy. This physicochemical properties are known as the Lipinski's rule of five because values of all the parameters are multiples of 5; the molecular weight should be less than 500 daltons, the calculated octanol-water partition coefficient (log P) should be less than 5, no more than five H-bond donors, and no more than ten H-bond acceptors.[26] For candidate molecule to be potentially used as drugs, it must meet the Lipinski's rule of five.[27,28] As shown in Table 3, all the *flavonoid* compounds did not meet the Lipinski rule, and therefore may not be orally active.

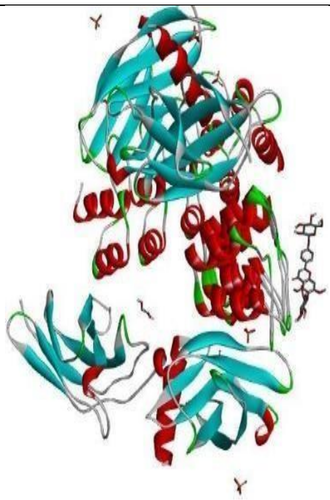
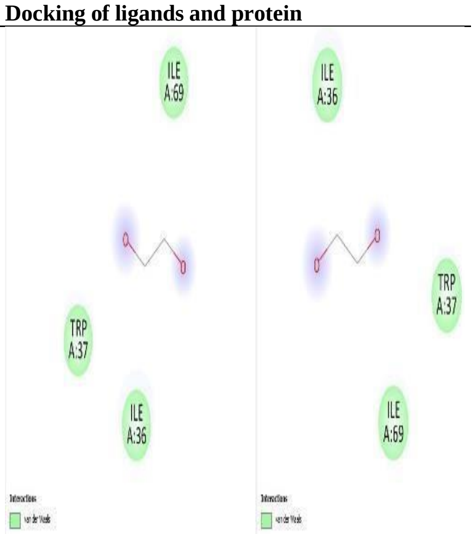
The result of the ADME predictions for *complanatuside* is presented in Table 4. The compound has a high absorption capacity as it was estimated to have an intestinal absorptionrate of over 80% (95.015%).

Table 5 shows the result of the toxicity prediction of the *flavonoid* compounds. The AMES toxicity test which is used to predict potentially mutagenic compounds shows negative result for all the test ligands, indicating that the ligands i.e. the *flavonoid* compounds are not mutagenic. Similarly, hepatotoxicity prediction shows negative result, indicating that the compounds are not hepatotoxic. Toxicity prediction based onmortality of Fathead minnow fish was presented as LC_{50} values. The compound *flavocommelin* had the lowest LC_{50} value (5.95 mM), suggesting that it is the most toxic of all the *flavonoid* compounds tested. *Complanatuside* had the lowest maximum tolerable dose in humans (0.248 mg/kg/day), *Kaempferol-3-O-gentiobioside* had the lowest acute oral toxicity in rats (2.48 mol/kg), while *Kaempferol-3-O-gentiobioside and Kaempferol-3-O-rutinuside* both had the lowest chronic oral toxicity in rats (3.56 mol/kg).

Table 1: Molecular Docking Score of the Interaction between ligands and target protein (EPO)

Ligand	**Ligand Type**	**Docking Score (kcal/mol)**	**Percentage (%)**	**RMSD (Å)**
ACI	Native ligand	-1.35	100	0.505
Flavocommelin	Test Ligand	-3.20	237.03	
Complanatuside	Test Ligand	-5.01	371.11	
Isoschaftoside	Test Ligand	-3.15	233.30	
Kaempferol-3-Gentiobioside	Test Ligand	-1.25	9.26	
Kaempferol-3-O-rutinuside	Test Ligand	-4.01	297.03	
Spinosin	Test Ligand	-2.69	199.25	
Fe	Control ligand	-4.37	100	0.478

Table 2: 3D visualization of molecular docking between ligands and target protein (EPO)

No	**Ligand**	**3D visualization**	**Docking of ligands and protein**
1	*Flavocommelin*		

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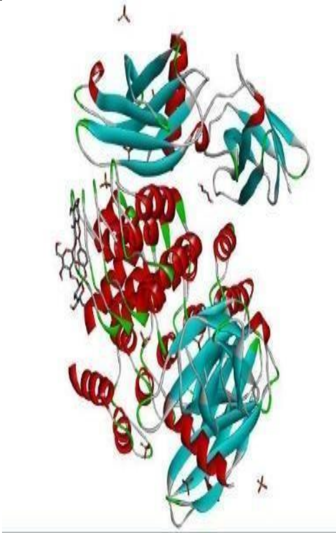
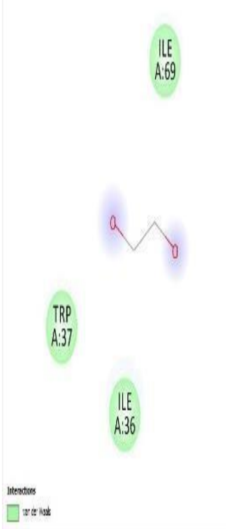
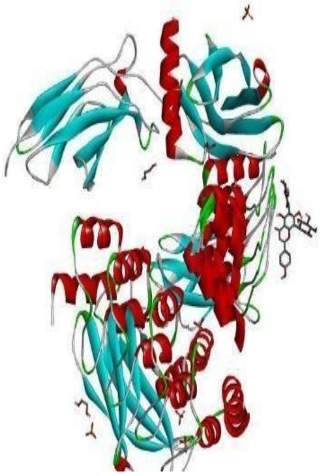
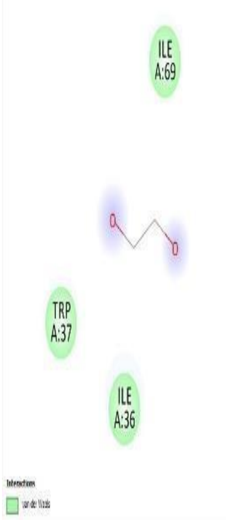
2 *Complanatuside*

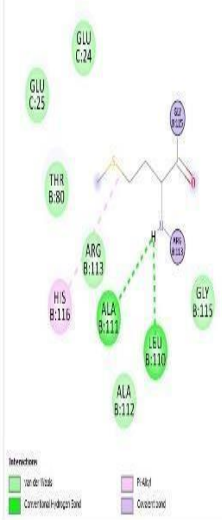




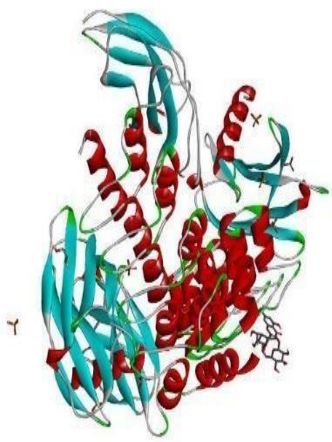
3 *Isoschaftoside*

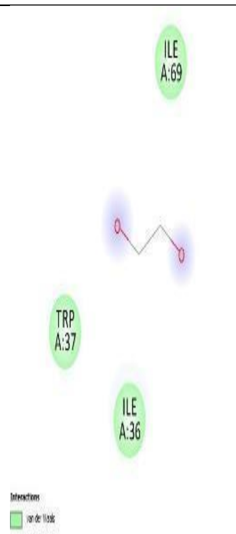


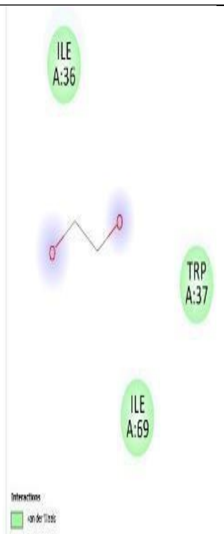




4 *Kaempferol-3-gentiobioside*







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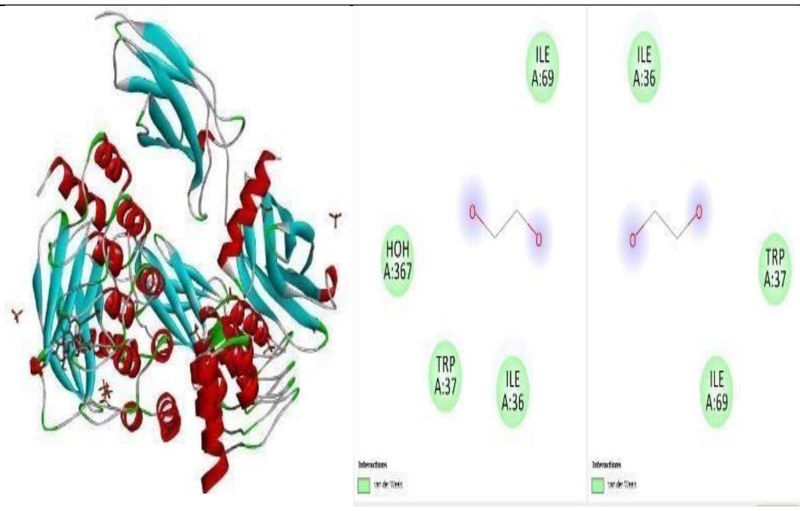
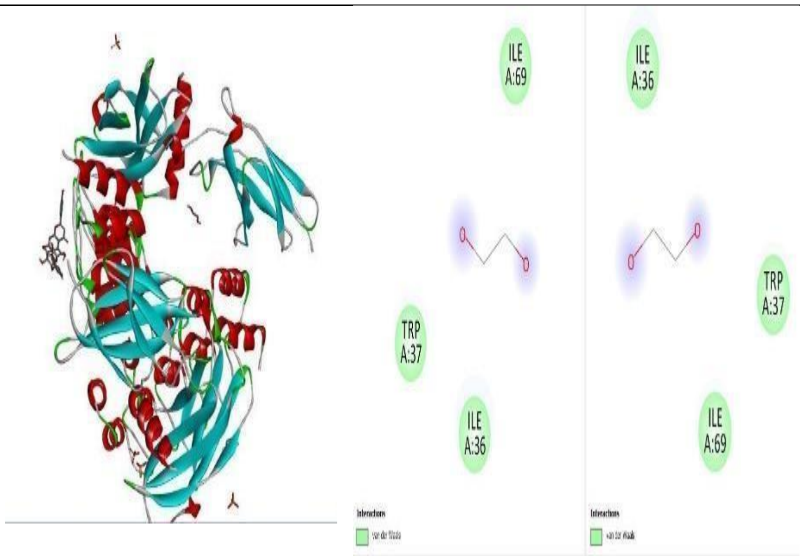
5	*Kaempferol-3-O-rutinuside*	
6	*Spinosin*	

Table 3: Physicochemical properties of the test ligands

Compound	Formula	Molecular weight (g/mol)	H-bond acceptor < 10	H-Bond donors < 5	Log P < 5
Flavocommelin	$C_{28}H_{32}O_{15}$	608.54	15	9	1.38
Complanatuside	$C_{28}H_{32}O_{16}$	624.54	16	9	1.23
Isoschaftoside	$C_{26}H_{28}O_{14}$	564.49	14	10	1.64
Kaempferol-3-gentiobioside	$C_{27}H_{30}O_{16}$	610.52	16	10	1.80
Kaempferol-3-O-rutinuside	$C_{27}H_{30}O_{15}$	594.52	15	9	1.13
Spinosin	$C_{28}H_{32}O_{15}$	608.54	15	9	1.07

Table 4: ADME predictions of complanatuside

ADME	Parameter	Outcome/Remark
A: Absorption	GI absorption	Low
	TPSA	250.97 A
	Bioavailability score	0.17
D: distribution	BBB	Not
	P-GP substrate	Yes
M: Metabolism	CYP1A2 inhibitor	Not
	CYP2C19 inhibitor	Not

	CYPC9 inhibitor	Not
	CYP2D6 inhibitor	Not
	CYP3A4 inhibitor	Not
E: Excretion	Log Kp	-11.30 cm/s

TPSA = Topological polar surface area

Table 5: Predicted toxicity of *flavonoid* compounds from *Ajwa date* fruits

S/N	Model Name	*Flavocomelin*	*Complanatuside*	*Isoschaftoside*	*Kaempferol-3-O-gentiobioside*	*Kaempferol-3-O-rutinuside*	*Spinosin*
1	AMES toxicity	No	No	No	No	No	No
2	Hepatotoxicity	No	No	No	No	No	No
3	Minnow toxicity (mM)	5.955	7.93	9.33	11.86	6.25	7.92
4	Max. tolerable dose in humans (mg/kg/day)	0.349	0.248	0.464	0.486	0.481	0.48
5	Acute oral toxicity in rats (mol/kg)	2.53	2.51	2.85	2.48	2.56	2.52
7	Chronic oral toxicity in rats (mol/kg)	6.138	6.19	5.08	3.56	3.56	4.81

Conclusion

In silico study of the *flavonoid* compounds from *Ajwa date fruit* extract showed that the *flavonoids* have potential antianaemic effect. All the compounds had good docking scores with the compound *complanatuside* having the most excellent binding affinity (-5.01 kcal/mol), which is comparable to that of the standard drug Fe with binding affinity of -4.37 kcal/mol. Althogh, the physicochemical properties did not obey the Lipinski rule of five, the *flavonoid* compounds haad a good pharmacokinetic profile. The toxicity prediction showed that the compounds are not toxic, and could be safe for consumption. Although, the *flavonoid* compounds have shown potential for antianaemic properties, further *in vitro* and *in vivo* studies is required to substantiate this claim.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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