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An Insight into the Precise Molecular Interaction and Inhibitory Potential of Amentoflavone and Its Substituted Derivatives on Human α-amylase

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Authors' contributions

This study was carried out in collaboration between both authors. Author THO designed the study, carried out the docking experiment and analysis as well as wrote the first draft of the manuscript. Author FCA conducted the literature review and arranged the data, managed the second draft and proofread the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Biflavonoids have attracted attention of researchers over the last few decades due to their pharmacological activities. Amentoflavone, a biflavonoid considered as apigenin dimer and isolated from various plants, is known for its large range of bioactivities. In the present study, we performed *in silico* experiment to corroborate the earlier wet experiment, that amentoflavone has anti-diabetic effect via inhibition of α-amylase enzyme. We also evaluated some naturally-occurring substituted derivatives of this compound as potential inhibitors of the enzyme.
Methodology: Molecular docking of the ligands on human α-amylase was determined by Vina plugin in PYMOL 1.3 and compared with acarbose, a known inhibitor of the enzyme.
Results: Our results showed a high binding affinity (-11.6 kcal/mol) for amentoflavone compared to

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acarbose (-9.3 kcal/mol) and a reliable configuration on the putative binding site of human α-amylase which can inhibit the enzymatic activity. Molecular analysis results revealed that amentoflavone made hydrophilic interactions with amino acid residues Asp300, Glu233, Asp197, His201 and Gln63 that are critical for catalytic activities of the enzyme. Comparison with its docked monoflavonoid subunit (apigenin) having a lower affinity (-8.9 kcal/mol) and 'partial' binding pose validates the possible inhibitory potential for amentoflavone. Some of the substituted derivatives such as bilobetin exhibited different poses within the binding pocket with lower affinity (-11.2 kcal/mol) compared to amentoflavone. The least binding affinity was observed for kayaflavone and heveaflavone with energy value of -9.9 kcal/mol.

Conclusion: The results of the current study suggest that amentoflavone inhibits human α-amylase competitively by occupying the substrate binding site and hindering substrate access. Our result is in agreement with the earlier wet experiment on the inhibitory properties of amentoflavone against human α-amylase. Taken together, this work provides insight into the precise molecular interaction of amentoflavone with α-amylase and validates the previous claims that the biflavonoid inhibit the enzyme as part of the mechanism for its antidiabetic activities.

Keywords: Amentoflavone; α-amylase; biflavonoid; diabetes; molecular interaction.

1. INTRODUCTION

Amentoflavone is a common biflavonoid which is found in medicinal plants across the globe. It is a dimeric form of apigenin containing a covalent C3' - C8" linkage [1]. According to literature, Okigawa and coworkers were the first researchers to isolate this compound from the *Selaginella* species such as *S. tamariscina, S. nipponica* and *S. pachystachys* [2]. As a polyphenolic compound, amentoflavone has been identified in a large number of plants. Till date, researchers have named more than 120 medicinal plants from which this biflavonoid has been isolated. Interestingly, most of these plants are traditionally used in folklore medicines. Few of such medicinal plants include *Selaginella tamariscina, Ginkgo biloba, Ouratea multiflora, Ginkgo subelliptica, Hypericum perforatum, Semecarous anacardium, Chamaecyparis obtusa, Juniperus phoenica, Selaginella doederleinii, Xerophyta plicata, Selaginella sellowii, Garcinia livingstonei, Ochna schweinfurthiana, Ouratea stipulata* and, *Cupressuceae spp, Podocarpaceae, Euphorbiaceae, Calophyllaceae* plant families [1, 3]. Generally, biflavonoids have been described as the dimeric form of monoflavonoids, which can be homo or hetero in combination, and are connected with a C-O-C or C-C bonds [4,5]. Although more than a hundred biflavonoids have been isolated and structurally described till date, medicinal plants that are known to contain them as main constituents are not so widely distributed [4]. However, most of the plants have been reported for various pharmacological potentials in different countries. The possible subunits that are commonly linked together to form biflavonoids are flavone-flavone, flavanone-flavone and flavanone-flavanone subunits while the connecting linkages are found at diverse positions [5]. The derivatives are formed by various substitutions made at different positions. The diversity observed among the biflavonoids is occasioned by the possibility of many different combinations which often results in various chemical structures.

Amentoflavone has been proved for numerous pharmacological activities [1,5]. Since its first isolation in 1971, amentoflavone has been extensively studied for a large range of bioactivities. Abdallah et al. observed the anti-inflammatory, anti-oxidation activities of amentoflavone in *in vitro* experiments [6]. The cytotoxic effect of this compound was reported by Ndongo and colleagues on various cancer cell lines [7]. In an *in vitro* screening experiment, Coulerie et al. found that amentoflavone exhibited anti-viral potential [8]. The biflavonoid displayed antibacterial as well as antifungal activity against several pathogenic fungal strains *in vitro* [9-11]. The antimalarial effect of amentoflavone is known while its therapeutic effects on cardiovascular system as well as the central nervous systems (CNS) have also been documented [12-14]. It has also been reported that amentoflavone possessed antihyperlipidemic and hepatoprotective properties [15-18]. Other reported bioactivities of amentoflavone include neuroprotection [11,19], anti-ulcerative colitis [20], antiangiogenic [17,21], anti-senescence [22], anti-psoriasis [23], anti-hypertrophic scar [24], antidepressant and radioprotection [25].

The antagonistic effect of amentoflavone on some human proteins/receptors has be documented in literature. For instance, amentoflavone reportedly inhibited phosphodiesterase isozyme 3 (PDE3) found in adipose tissue as well as phosphodiesterase 5 (PDE5) [3,26]. The biflavonoid also inhibited acetylcholinesterase [27,28], cytochrome CYP3A4, CYP2C9 [29], nitric oxide synthase and fatty acid synthase [30]. Lee and coworkers [5] reported that amentoflavone is an allosteric inhibitor of protein tyrosine phosphatase 1B (PTP1B) in *in vitro* and *in silico* experiments. In a related manner, Hanraha and colleague discovered that the compound is an antagonist of kopioid receptor [31] while Colovic et al. claimed that the compound is an inhibitor of human cathepsin B(1) [32]. Amentoflavone was also said to modulate benzodiazepine GABA (A) receptor at the allosteric site, inactivate NF-kß [32] and block the enzymatic activity of vascular endothelial growth factors [33,34].

Natural molecules (including plant-derived polyphenolic compounds like amentoflavone) are perhaps more acceptable as source of antidiabetic agents, compared to the synthetic drugs [35]. As a means of successfully lowering postprandial hyperglycaemia, glucose absorption is inhibited by blocking the enzymes involved in carbohydrate hydrolysis. Known inhibitors, such as acarbose, delay carbohydrate digestion resulting in a reduced rate of glucose uptake and absorption. Albeit these drugs are effective, they have been reported to exhibit adverse effects such as flatulence, diarrhea, constipation and severe stomach pain [36]. Thus, natural products are sought to replace these pharmaceutical agents because they are believed to be relatively cheap, less harmful and effective. Since α-amylase has become one of the clinical targets in the management of diabetes due to its key role in carbohydrate digestion, the enzyme is being targeted for inhibition, especially in type-2 diabetes, to retard the breakdown and absorption of glucose [37]. This strategy is useful because the time required for increasing blood glucose level becomes extended to span a longer period with gradual release of glucose from dietary source in diabetic patient. Due to the high cost as well as toxicity of insulin and other synthetic antidiabetic drugs such as acarbose, researchers have explored available medicinal plants with antidiabetic and hypoglycemic effects as sources of cheaper and less toxic alternatives [37,38]. Some of the plants with such potentials are found to contain biflavonoids including amentoflavone.

According to recent report, amentoflavone, a naturally-occurring biflavonoid obtained from many medicinal plants, inhibited activity of α-amylase in *in vitro* experiment [15]. Here, we carried out docking to evaluate the precise molecular interaction as well as inhibitory potential of amentoflavone and its substituted derivatives on α-human amylase. The results will elucidate the antidiabetic potential as well as the interaction signatures of this compound and may aid the design and development of effective antidiabetic drug candidate using α-amylase as a validated target.

2. MATERIALS AND METHODS

2.1 Selection and Preparation of Protein Structure through Homology Modeling

The starting coordinate of α-amylase used in this study were retrieved from the Brookhaven protein data bank (http://www.rcsb.org/pdb) with PDB ID: 2QV4 having resolution of 1.97 Å. The crystal structure was deposited by Maurus et al. in 2007 [39]. α-amylase was co-crystallized with acarbose. The "FASTA" files (Accession: 2QV4_A GI:170785004) for the α-amylase was retrieved from www.pubmed.org and used in homology modeling as done on the Swiss-Model Server (http://swissmodel.expasy.org). The active site of the macromolecules was identified with reference to the co-crystallized ligand which was deleted, in addition to the crystallographic water molecules, from the protein before molecular docking procedures.

2.2 Ligands Preparation and Optimization

A total of twelve (12) ligands used in this docking study were selected from the literature. Out of these compounds, ten (10) were biflavonoids isolated from numerous medicinal plants while the other compounds (acarbose and apigenin) served as control ligands. The chemical structures of the plant-derived biflavonoids: amentoflavone (CID: 5281600), bilobetin (CID: 5315459), sequoiaflavone (CID: 5484010), ginkgetin (CID: 5271805), isoginkgetin (CID: 5318569), putraflavone (CID: 5320646), heveaflavone (CID: 15559724), kayaflavone (CID: 9894522), sciadopitysin (CID: 5281696) and sotetsuflavone

(CID: 5494868) were obtained from NCBI PubChem compound database (http://www.ncbi.nlm.nih.gov/pccompound) and prepared using Marvinsketch. 2D-coordinates of the ligands were sketched using ChemAxon software (https://www.chemaxon.com) and, using the Conformers suit of Marvin-Sketch, the 2D structures were converted to 3D geometry. The Merck molecular force field (MMFF94) was employed. The .sdf format of the compounds were docked into the targets using AutoDock 4.2.

2.3 Validation of Molecular Docking Procedure

One of the major ways of validating docking procedure is to accurately regenerate both the pose and the molecular interaction of the co-crystallized ligand on the crystallographically determined protein structure [40]. The ligand found at the binding site of the experimentally determined α-amylase was deleted. The structure of the ligand (.sdf format) was separately prepared using Marvin sketch as described above and re-docked into α-amylase active site. The molecular interaction, majorly hydrogen bond in this case, was compared to that of the x-ray diffraction crystal structure.

2.4 Molecular Docking and Scoring

For ligand docking and target-ligand complex analysis, Autodock vina suite on PYMOL [41,42] was used. First, based on the already present co-crystallized ligand in the pdb file, the inhibitor binding site was defined with grid parameters set at x=100, y=100 and z=100 while the coordinate of origin (x, y and z) was set at 12.38, 48.14 and 26.21 to include all the amino acid residues at the active site. This gives enough space to enhance adequate ligand rotation and translation. The spacing between grid points was maintained at 0.375 angstroms. All optimized ligands were docked to the active site of the proteins. While the rotatable bonds of the ligands were set to be free, the protein molecules were treated as rigid structures [43]. Throughout this *in silico* investigation, ten (10) docking runs were performed for each ligand with the number of modes set to 10 so as to achieve more accurate and reliable results.

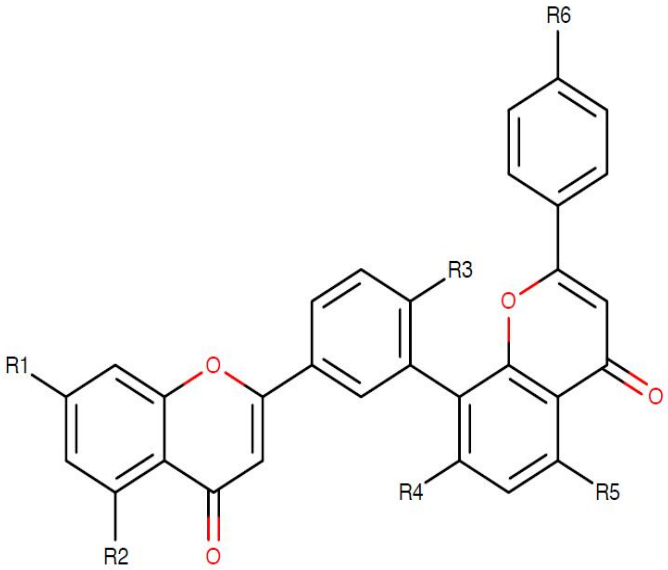
2.5 Data Analysis

The protein-ligand complexes as well as the molecular interaction were all visualized using PYMOL and snapshots were taken. Ligplot was used to depict details of protein-ligand interactions [44].

3. RESULTS AND DISCUSSION

In this study, computational experimental procedure was adopted to elucidate the precise molecular interaction, binding configuration and inhibitory potential of amentoflavone, a plant-derived biflavonoid, and its substituted derivatives as potential inhibitors of human α-amylase. This research seeks to corroborate previously reported wet experiments that amentoflavone is a potent inhibitor of α-amylase and, also investigate its naturally-occurring derivatives as antidiabetic compounds. The molecular docking experiment was done using AutoDock 4.2 with PYMOL. The protein structure used as target for the docking simulation was modelled on Swiss Model server based on the template retrieved from PDB database. The docking protocols was first validated to ensure quality and reliability of the docking results obtained. We selected human α-amylase crystal structure with PDB ID: 2QV4 from the protein data bank as the starting coordinate for our target. To date, several crystal structures of human α-amylase, co-crystallized with different inhibitors, have been deposited in the protein database. Our program successfully regenerated the ligand binding conformational geometry in a similar pattern to the experimental results (Fig. 3). Both the binding poses and molecular interactions found in the crystallographic human α-amylase structure were accurately reproduced suggesting the acceptability of our docking protocols [45]. Fig. 1 shows the structure of amentoflavone and its derivatives that were employed in this study. The subtle structural differences found among the biflavonoids may accounts at least in part for the varied binding pattern, affinity, potency and molecular interaction of the compounds to their target. Fig. 2 displays the reference ligand (acarbose) as docked to the active site of human α-amylase. The ligand is surrounded extensively by amino acid residues Ile235, His305, Asp300, His299, Trp58, Trp59, Glu233, His201, His101, Arg195, Ala198, Thr163, Ile51, Asn105, Gly164, Val107, Gln63, Gly104, Tyr62, and Leu165 within 4 Å at the active site. Seven complex hydrogen bonds were established with amino acids which have been tagged as the critical residues for the enzyme inhibition [46]. Acarbose, a pseudosubstrate, inhibits α-amylase competitively via interaction with the active site. Hence, it is used

to lower postprandial blood glucose level in type-2 diabetes [47]. The estimated binding energy value obtained for acarbose in the current study is -9.5 kcal/mol which is relatively lower than the value reported by Metibemu et al. [48].



S/No	Name	R_1	R_2	R_3	R_4	R_5	R_6
1	Amentoflavone	OH	OH	OH	OH	OH	OH
2	Bilobetin	OH	OH	OCH_3	OH	OH	OH
3	Sequoiaflavone	OCH_3	OH	OH	OH	OH	OH
4	Ginkgetin	OCH_3	OH	OCH_3	OH	OH	OH
5	Isoginkgetin	OH	OH	OCH_3	OH	OH	OCH_3
6	Putraflavone	OCH_3	OH	OH	OH	OH	OCH_3
7	Heveaflavone	OCH_3	OH	OH	OCH_3	OH	OCH_3
8	Kayaflavone	OH	OH	OCH_3	OCH_3	OH	OCH_3
9	Sciadopitysin	OCH_3	OH	OCH_3	OH	OH	OCH_3
10	Sotetsuflavone	OH	OH	OH	OCH_3	OH	OH

Fig. 1. Chemical structure of amentoflavone and its substituted derivatives

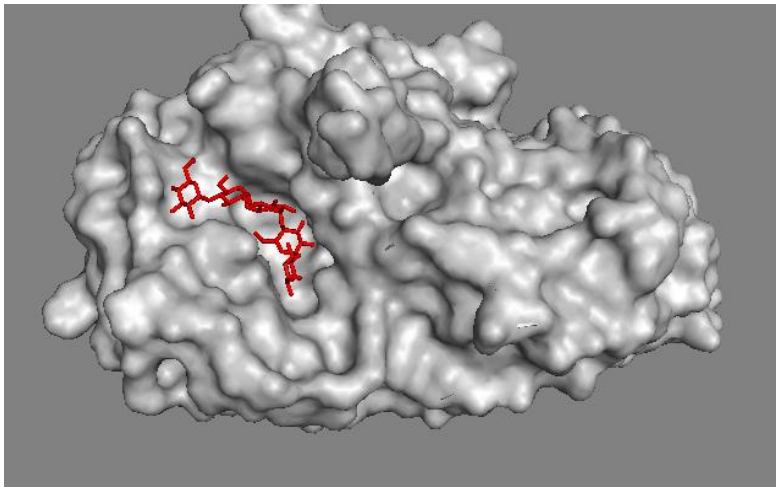


Fig. 2. Human α-amylase enzyme (PDB: 2QV4) with acarbose at its active site

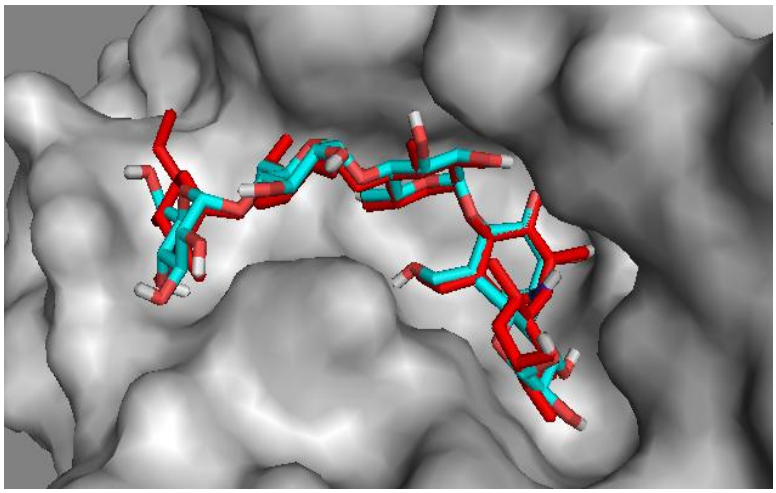


Fig. 3. Acarbose, control ligand, on human α-amylase (PDB: 2QV4) active site; red stick represents co-crystallized ligand while blue stick represents re-docked ligand with Autodock

Table 1. Binding energy and hydrogen bond interaction of amentoflavone and its derivatives

S/No	Ligand	Energy value (kcal/mol)	Amino acid residues involved in Hydrogen bond
1	Amentoflavone	-11.6	Gln63, His201, Asp197, Asp300, Glu233
2	Bilobetin	-11.2	Gln63, His299, Asp197, Tyr151, Asp300, Arg195
3	Sequiaflavone	-11.5	Asp300, Asp197, Gln63, Glu233, His201
4	Gingketin	-11.3	Arg195, Gln63, Tyr151, Asp300
5	Isoginkgetin	-10.4	His201, Asp197, Gln63
6	Putraflavone	-11.4	Glu233, Asp197, His201, Gln63, Asp300
7	Heveaflavone	-9.9	His299, Gln63, Tyr151
8	Kayaflavone	-9.9	Gln63, Tyr62, Glu233
9	Sciadopitysin	-10.3	Asp197, His201, Gln63
10	Sotetsuflavone	-11.3	Tyr151, Gln63, Tyr151, Asp300
11	Apigenin	-8.9	Gln63, Arg195, Asp197, Asp300
12	Acarbose	-9.5	Gln63, His101, Ala106, Thr163, Gly164, Arg195, His201, Glu233, His299, Asp300

The binding energy values obtained for the bifavonoids in this study are presented in Table 1. Among these compounds, amentoflavone has the highest affinity (lowest binding energy) to the target. A detailed observation of the interaction between amentoflavone and α-amylase revealed that the compound completely occupied the substrate binding site (Fig. 4). The docking energy and pose of amentoflavone was compared between the crystallographically determined structure and the modeled protein structure. The result, as shown in Fig. 4, revealed a similar pose, binding energy value (-11.6 kcal/mol) and hydrogen bond interaction for amentoflavone on the target. This contributes, in a way, to the validation and acceptability of our docking model used in this study. Also, the binding pose of amentoflavone was found to be similar to that of acarbose, the control ligand. We further compared the binding profile of apigenin ('monomer' of the amentoflavone) to that of amentoflavone by superimposing amylase-amentoflavone complex to amylase-apigenin complex (Fig. 5). Interestingly, a comparable binding pose was obtained for the monoflavonoid and a subunit of the biflavonoid suggesting that amentoflavone used its monoflavonoid subunits to interact with the enzyme. However, apigenin showed a reduced affinity (-8.9 kcal/mol) to the enzyme compared to its dimeric form and acarbose (Table 1). Both the biflavonoid and its 'monomer' commonly displayed hydrogen bonds with residues Gln63, Asp197 and Asp300. In addition to these interactions, apigenin established extra hydrogen bond with Arg195 while amentoflavone formed additional hydrogen

bonds with His201 and Glu233 (Fig. 5). The presence of another apigenin subunit in the biflavonoid may have enhanced its interaction with the target. This is in agreement with previous claims that biflavonoids possess higher biological activities compared to the monoflavonoids [49,50]. The presence of dimeric structure of apigenin which makes up amentoflavone appears to enhance its interaction as well as the inhibitory potential on the enzyme at the active site. Amentoflavone is found in the leaves and whole plant of many medicinal plants and may account at least in part for their pharmacological properties including antidiabetic activity [51,52].

Results obtained from the molecular interaction analysis show that amentoflavone established hydrophilic interaction with five distinct amino acid residues such as Glu233, Asp197, Asp300, Gln63 and His201. These residues are very critical for the catalytic activity of the enzyme. Marek et al. [46] had earlier reported that amino acid residues Glu233, Asp300 and Asp197 participate in the enzymatic steps involved in conversion of the substrate (carbohydrate) to glucose. The residues were also seen in the inhibitory interaction between the enzyme and acarbose. Hydrophobic interactions with His305 and Trp59 were also observed. These interactions contribute to the enzyme-ligand complex stability. Meanwhile, in evaluating a protein-ligand complex, attention is often placed on hydrogen bond interaction because of its key roles in catalysis and structural stability of biological system. Hydrogen bond is also one of the ubiquitous elements for molecular recognition in biological systems and, based on reported observation from the PDB structures, the expected length of hydrogen bonds often ranges from 2.6 to 3.1 Å. As seen in all Ligplot depictions, the length of hydrogen bond obtained in this study fall within this range. Previously, various extracts of medicinal plants which contain biflavonoids have been reported to show potent biological activities. In fact, various extracts of amentoflavone-containing plants have been shown to exhibit antihyperglycemic potential [53-56]. In a related report, amentoflavone was reported to block enzymatic activity of human α-amylase [15]. Hence, our results are compatible with earlier *in vitro* and *in vivo* experiments [15,55]. Since α-amylase is one of the major enzymes associated with carbohydrate metabolism, inhibition of this enzyme by amentoflavone may lead to reduction in glucose absorption which ultimately normalizes the blood glucose level in type-2 diabetes.

As given in Fig. 6, the predicted binding poses of bilobetin and kayaflavone which were chosen as samples of the substituted derivatives of amentoflavone showed varied binding configurations on the enzyme active site. Also, their binding affinity was lower compared to that of amentoflavone (Table 1). The docked bilobetin appeared to have been rotated at the putative binding site compared to the control ligands (acarbose and apigenin) and amentoflavone (Fig. 6). Hydrogen bonds were seen with residues Tyr151, Gln63, His299, Asp300, Asp197 and Arg195 as established by bilobetin. On the other hand, kayaflavone preferably formed hydrogen

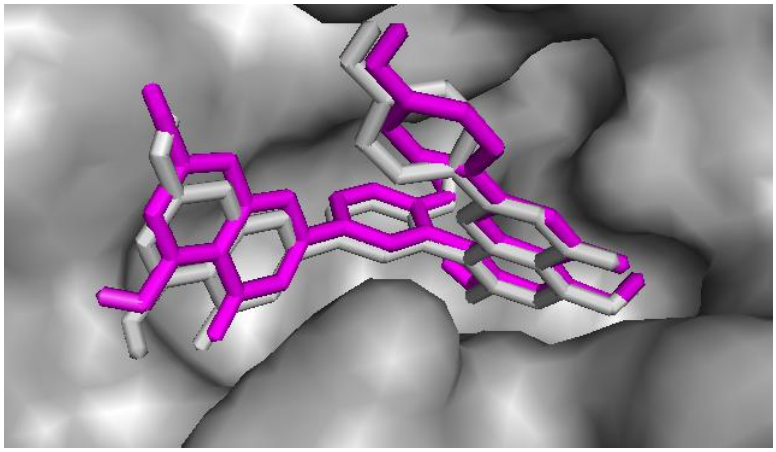


Fig. 4. Superimposed docked amentoflavone on α-amylase crystal (PDB:2QV4; white stick) versus modeled structure (magenta) showing comparable binding configuration. The validates and also enhances the reliability and acceptability of the protein model used in this study

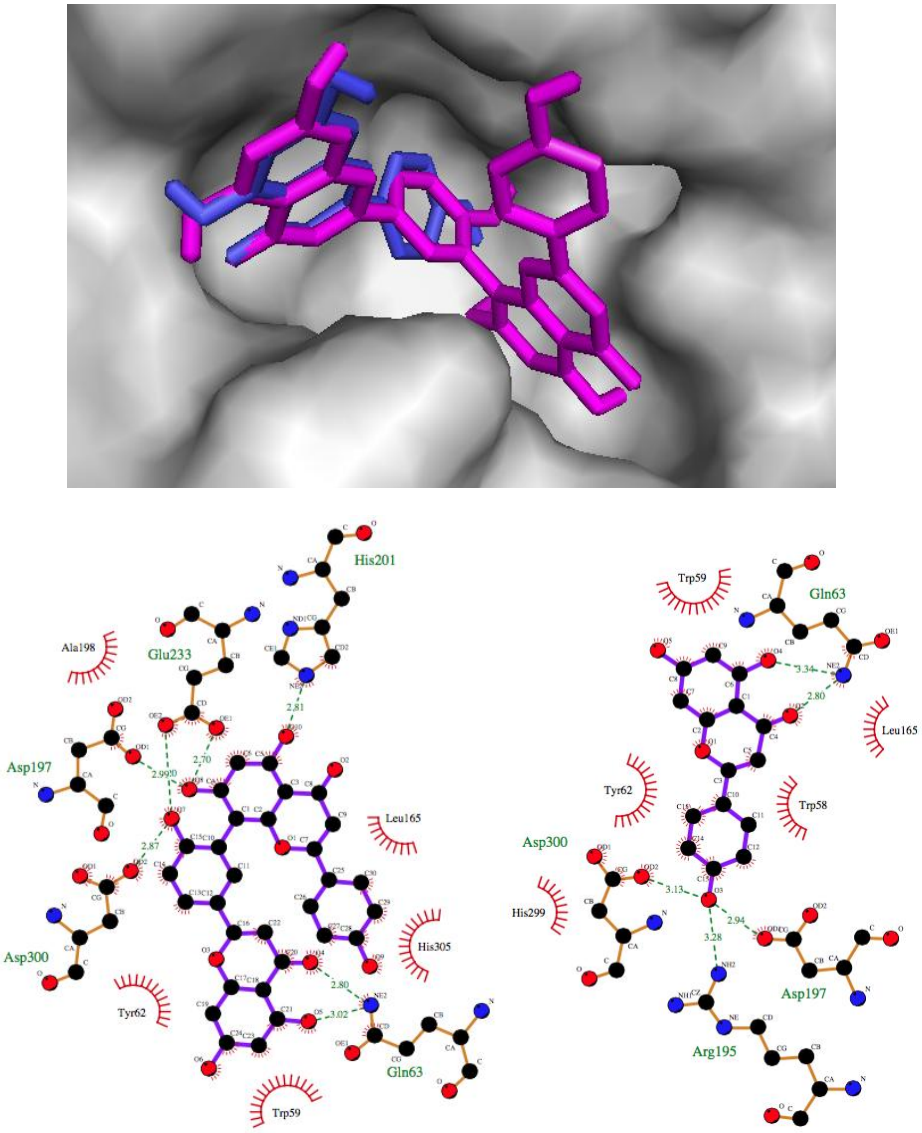


Fig. 5. Comparative analysis of docking pose and molecular interaction of amentoflavone (magenta) versus apigenin (blue) on α-amylase active site

bonds with three (3) amino acid residues, Tyr62, Gln63 and Glu233. The reduced number of hydrogen bond may account for the lower affinity of kayaflavone towards the enzyme at the active site [57]. In addition, the binding configuration of this compound differed from that observed for both amentoflavone and acarbose (Fig. 6). However, these compounds displayed potential to occupy the active site with a possibility of preventing substrate access. Bilobetin is obtained from *Ginkgo biloba* leaves and seeds as an active polyflavonoid [58,59]. The plant has long been employed as a traditional Chinese medicine to treat several human diseases including diabetes and hyperlipidaemia. Kou and coworkers had earlier reported that bilobetin ameliorates insulin resistance in rats fed a high-fat diet [60].

Although biflavonoids have been studied for numerous biological and pharmacological activities, only few of them are investigated in diabetes and its complication. Among the few biflavonoids are amentoflavone and kolaviron [61]. This research therefore provides an insight into the possible antidiabetic potential of the derivatives of amentoflavone and may lend credence to the yet untapped α-amylase

inhibitory properties of the biflavonoids. From our results, it is obvious that the linkage type of a biflavonoid is a determining factor for them to gain precise molecular interaction pattern that can inhibit α-amylase as antidiabetic target. The different positions of the hydroxyl and methoxyl moieties found on the biflavonoids also appear to influence their fitness, binding pose as well as interaction within the binding pocket which eventually determines their inhibitory potential against the enzyme. Amentoflavone, bilobetin and the other substituted derivatives are biflavone linked at 5',8" linkage position. Amentoflavone is 5',8"-biapigenin while bilobetin is a 4'-O-methylamentoflavone [5].

Taken together, ametoflavone, a plant-derived biflavonoid, has the best inhibitory potential out of all the biflavonoids chosen for this research (Fig. 8). Nevertheless, the substituted derivatives of amentoflavone may show tendency to block α-amylase catalytic activity competitively. Considering the bulky hydrophobic structure of the biflavonoids, the contribution of van der waals interactions as well as hydrophobic interactions to the binding strength observed between α-amylase and the ligands is not improbable. The difference in binding pose and molecular interaction potential among the biflavonoids may play a role in the variations observed in their potency and bioactivities.

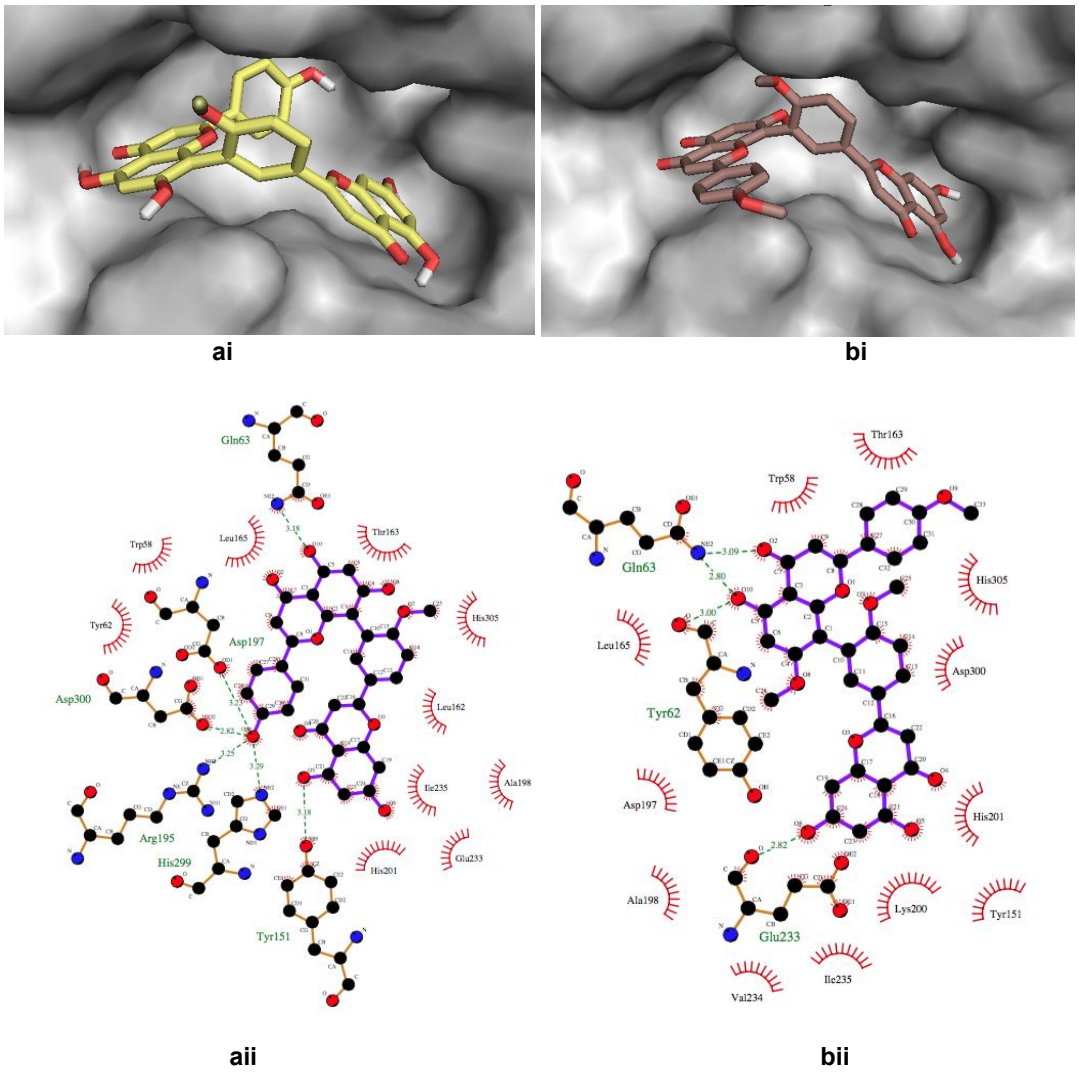


Fig. 6. Binding orientation and molecular interaction analysis of (a) bilobetin versus (b) kayaflavone on α-amylase active site

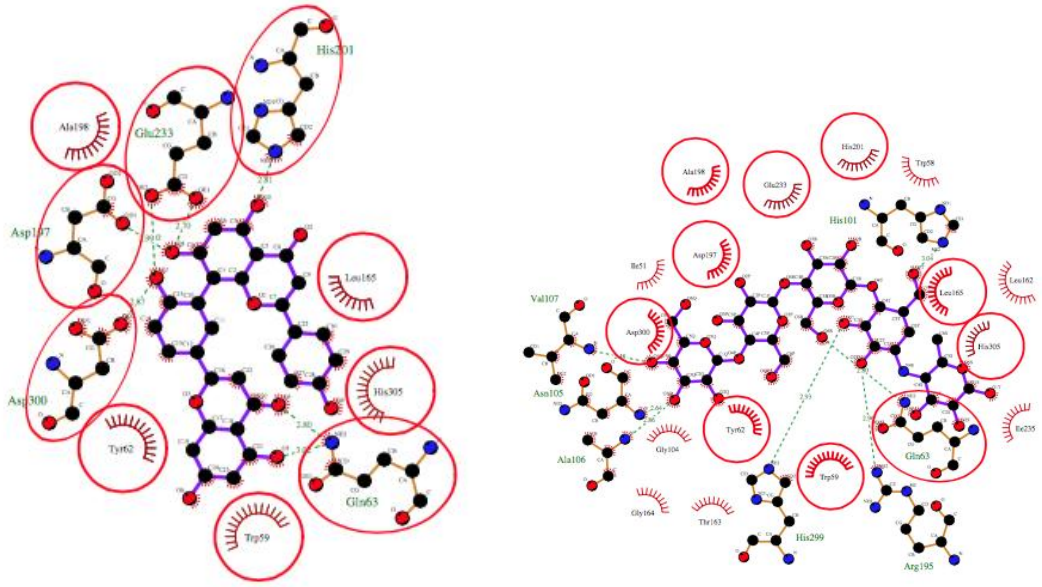


Fig. 7. Comparative analysis of molecular interaction of (c) amentoflavone and (d) acarbose

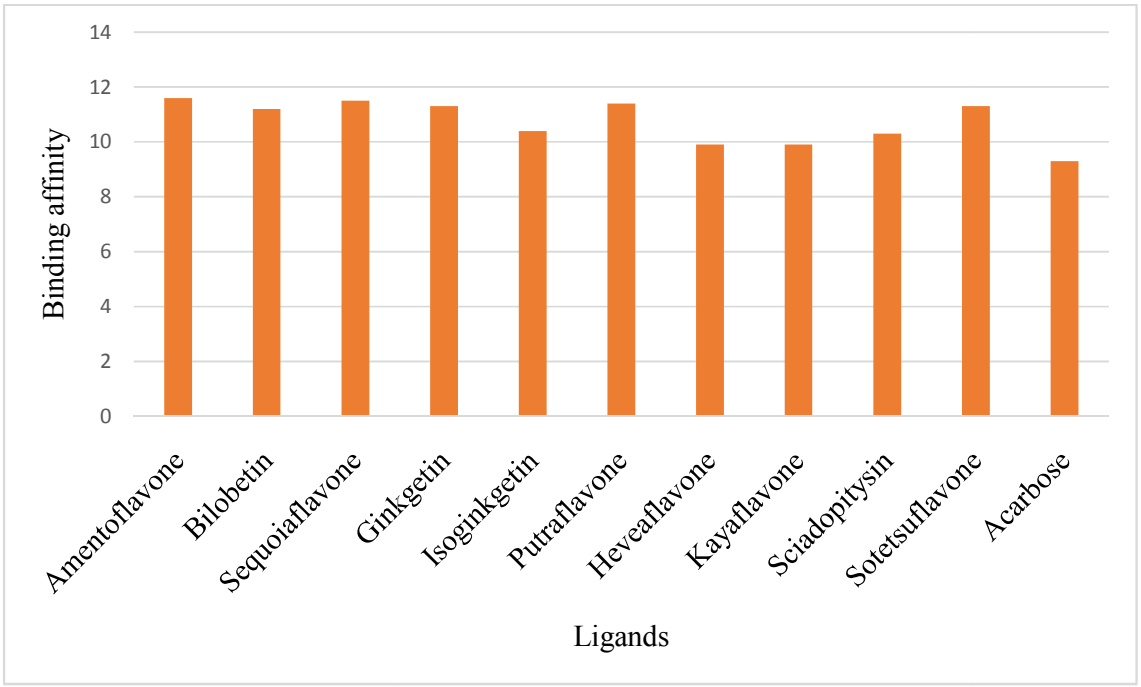


Fig. 8. α-amylase inhibitory activity of biflavonoids based on docking studies

4. CONCLUSION

In this study, we determined the molecular interaction and inhibitory potential of amentoflavone and its derivatives on human α-amylase using *in silico* approach. Our results confirmed that amentoflavone was embedded and fitted well into the binding pocket where it can hinder substrate binding competitively. Compared to acarbose, the biflavonoid has

higher affinity to the enzyme at the active site. This indicates the possible greater inhibitory potential of the biflavonoid. We also comparatively docked the monoflavonoid, apigenin to the enzyme and found a similar binding pose compared to amentoflavone. This supports the validation of the docking model as well as the inhibitory potential of the biflavonoid. However, the monoflavonoid showed reduced affinity to the target. Based on the docking model, we evaluated some derivatives of amentoflavone against the target and found them having lower binding affinity as well as altered configuration to the enzyme compared to amentoflavone. This result suggests that amentoflavone is the best inhibitor of the enzyme among the biflavonoids studied in this experiment. However, it should be noted that there still exists a dearth of data from wet experiment on the α-amylase inhibitory effect of the other biflavonoids. The biflavonoids are therefore suggested for *in vitro* evaluation as potential antidiabetic compounds. Notwithstanding, this study verified amentoflavone as a competitive inhibitor of human α-amylase and corroborates the previous wet experiment that amentoflavone possesses antidiabetic activity. The structure of the biflavonoids may be useful in the design and development of antidiabetic drug candidate targeting α-amylase.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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