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In Vitro and *In Silico* Study on the Interaction between Apigenin, Kaempferol and 4-Hydroxybenzoic Acid in Xanthine Oxidase Inhibition

(Kajian Secara *In Vitro* dan *In Silico* pada Interaksi antara Apigenin, Kaempferol dan Asid 4-Hidroksibenzoik dalam Perencatan Xantina Oksidase)

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ABSTRACT

Xanthine oxidase (XO) is a biological enzyme that takes part in purine catabolism. It catalyses the conversion of hypoxanthine to xanthine and eventually xanthine to uric acid. The catabolism reaction increases the level of uric acid and subsequently leads to hyperuricemia. Allopurinol is a XO inhibitor that is used clinically to prevent purine catabolism. Although it is an effective XO inhibitor, it causes some side effects. Therefore, a more effective inhibitor with fewer side effects is in an urgent need. Phenolic compounds have been identified as effective XO inhibitors in many studies. *In vitro* and *in silico* study were conducted to investigate the interaction between apigenin, kaempferol and 4-hydroxybenzoic acid in XO inhibition. Apigenin was found to be the most effective XO inhibitor among the compounds tested with the best docking score of -8.2 kcal/mol as demonstrated in the molecular docking simulation which indicated its favourable interaction with XO enzyme. Additive interactions between compounds namely apigenin-kaempferol, apigenin-4-hydroxybenzoic acid and 4-hydroxybenzoic acid-kaempferol were demonstrated in both *in vitro* and *in silico* studies. The results showed that 4-hydroxybenzoic acid- apigenin (-7.4 kcal/mol) was the most stable ligands combination docked to XO. The multiple ligands docking simulation showed independent ligands bound to the XO active site at non-interfering regional location. In conclusion, the combination of these three compounds can be explored further for their additive interaction in XO inhibition, which could be beneficial in terms of the enhanced effectiveness and lower side effects when each is used at lower dose to give the same effect.

Keywords: Additive interaction; molecular docking; multiple ligands; phenolic compounds; xanthine oxidase inhibitor

ABSTRAK

Xantina oksidase (XO) ialah sejenis enzim biologi yang terlibat dalam metabolisme purin. Ia memangkinkan penukaran hipozantin kepada xantina dan akhirnya daripada xantina kepada asid urik. Tindak balas katabolisme meningkatkan tahap asid urik dan seterusnya membawa kepada hiperurisemia. Allopurinol adalah sejenis perencat XO yang digunakan secara klinikal untuk mencegah katabolisme purin. Walaupun ia adalah sejenis perencat XO yang berkesan, ia menyebabkan kesan sampingan. Oleh itu, perencat yang lebih berkesan serta kurang kesan sampingan adalah amat diperlukan. Sebatian fenolik telah dikenal pasti sebagai perencat XO yang berkesan dalam banyak kajian. Kajian *in vitro* dan *in siliko* telah dijalankan untuk mengkaji interaksi antara apigenin, kaempferol dan asid 4-hidrosibenzoik semasa perencatan XO. Apigenin didapati merupakan perencat XO yang paling berkesan dalam kalangan sebatian yang dikaji dengan skor dok yang terbaik sebanyak -8.2 kcal/mol sebagaimana yang ditunjukkan oleh simulasi dok molekul yang menunjukkan interaksi yang menggalakkan dengan enzim XO. Interaksi secara tambahan antara sebatian iaitu apigenin-kaempferol, apigenin-asid 4-hidroksibenzoik dan asid 4-hidroksibenzoik-kaempferol telah ditunjukkan dalam kajian *in vitro* dan *in siliko*. Hasil kajian menunjukkan asid 4-hidroksibenzoik-apigenin (-7.4 kcal/mol) adalah gabungan ligan yang paling stabil semasa didokkan pada XO. Simulasi dok berbilang ligan menunjukkan ligan bebas terikat pada tapak aktif XO di lokasi yang tidak mengganggu antara satu sama lain. Secara kesimpulannya, gabungan ketiga-tiga sebatian ini boleh diterokai dengan lebih lanjut dari segi interaksi tambahan mereka dalam perencatan XO, yang boleh dimanfaatkan dari segi peningkatan keberkesanan dan pengurangan kesan sampingannya dapat dipertingkatkan apabila setiap satu digunakan pada dos yang lebih rendah untuk memberikan kesan yang sama.

Kata kunci: Dok molekul; interaksi tambahan; pelbagai ligan; perencat xantina oksidase; sebatian fenolik

Introduction

Xanthine oxidase (XO) is an enzyme first discovered in bovine milk by Schardinger in 1902. It is a homodimer containing two subunits, forming a butterfly shaped complex structure. Each subunit contains one molybdenum, one flavin adenine dinucleotides (FAD) and two iron sulfur (2Fe-2S) (Figure 1). XO consists of three domains which are N-terminal, C-terminal and intermediate domain (Enroth et al. 2000). XO is an enzyme responsible for purine catabolism that converts hypoxanthine to xanthine and subsequently to the final product, uric acid (Lin et al. 2002). It results in a high level of uric acid in the human body when hypoxanthine is catalysed continuously. The normal range of uric acid level in human body is 2 - 3 mg/dL for female and 3 - 4 mg/dL for male. Uric acid level above 6 mg/dL in female and 7 mg/dL in male is regarded as hyperuricemia (Shani et al. 2016).

Overproduction or under excretion of uric acid can lead to gout disease, which is a common inflammatory arthritis (Ichide et al. 2012). The common symptoms of gout are inflammation, deposition of urate crystals in the joints and kidneys, gouty arthritis, and uric acid nephrolithiasis (Ng et al. 2022). Allopurinol is an effective clinical drug that is widely used to treat hyperuricemia and gout disease. However, it is associated with many side effects such as renal failure, impaired liver function, hypersensitivity syndrome, skin rash and allergic reactions (Murata et al. 2009). Therefore, a more effective inhibitor with fewer side effects is in an urgent need.

Drug discovery and development using computational methods are increasingly gaining in popularity recently. Among different computational methods, molecular docking has become an integral part of the drug discovery program. Molecular docking is a computer software that is normally used to predict the intermolecular forces between ligand and a protein for the determination of inhibitory activity (Tao et al. 2019). When a ligand binds to the active site of a protein, scoring function is used to predict the strength of the non-covalent interactions (Santoyo et al. 2013). The more negative the binding affinity, the lower the energy spent

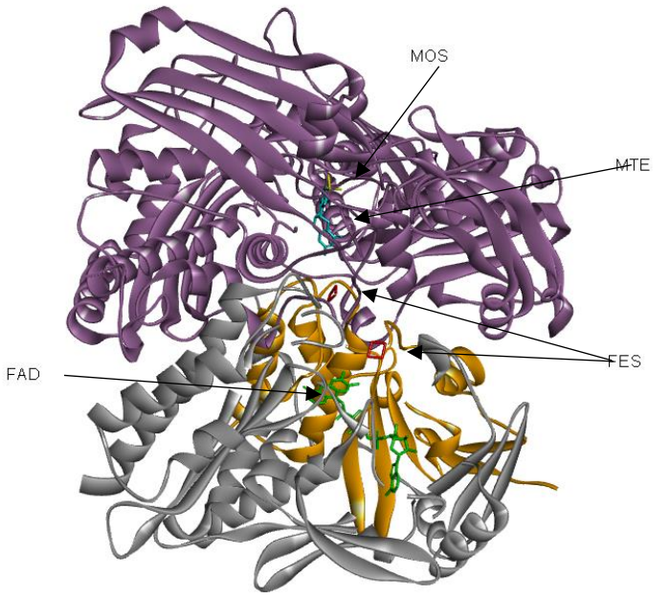

FIGURE 1. 3D structure of bovine xanthine oxidase monomer. Chain A, B and C are coloured in orange, grey and purple, respectively. The bound FAD (green), Fe-S cluster (red), MTE (blue) and MOS (yellow) are indicated. The xanthine oxidase crystal structure in complex with quercetin (3NVY) was downloaded from the RCSB Protein Data Bank (http://www.rcsb.org), the left side of the homodimer and the quercetin were removed using Discovery Studio visualizer

to bind to the protein, the more favorable is the binding mode (Du et al. 2016).

Polyphenols are plant secondary metabolites that possess antioxidant, anti-inflammatory, antibacterial, hypoglycemic and enzyme inhibitory properties (Liu et al. 2020; Xie et al. 2014; Zhao et al. 2018). They are widely studied due to their diverse biological properties and being consumed as natural remedies for the management of hyperuricemia. Polyphenols usually have aromatic rings with hydroxyl groups or other substituents (Liu et al. 2020). They are known to be good XO inhibitors due to the phenol portion and alkenyl chain (Masuoka & Kubo 2018). Flavonoids which are a family of polyphenolic compounds, have been identified as effective XO inhibitors in many studies (Lin et al. 2015a; Malik, Dhiman & Khatkar 2019). Apigenin and kaempferol have been shown to inhibit XO effectively by occupying the active site of the enzyme (Lin et al. 2002; Wang et al. 2015), whereas 4-hydroxybenzoic acid was shown to possess weak inhibition against XO (Masuda et al. 2013). Various combinations of flavones and flavonols have been shown to exhibit synergism. To the best of our knowledge, there is no study conducted on the interaction between flavonoids and phenolic acids on the inhibition of XO enzyme. Therefore, the objective of this research was to investigate the interaction among apigenin, kaempferol and 4-hydroxybenzoic acid on XO inhibition using *in vitro* and *in silico* method.

Materials and Methods

CHEMICALS

Analytical grade kaempferol, apigenin, 4-hydroxybenzoic acid, xanthine and allopurinol were purchased from Sigma Aldrich (St. Louis, United States). Xanthine oxidase was purchased from Roche Diagnostics (Basel, Switzerland). Sodium hydroxide pellets was purchased from Honeywell Riedel-de-Haën™. Dipotassium hydrogen phosphate was purchased from SYSTERM (Petaling Jaya, Malaysia) and potassium dihydrogen phosphate was purchased from Merck (New Jersey, United States). Ultrapure water from a Mili-Q® purification system was used in this research.

SOFTWARE

Autodock Tools-1.5.6 was downloaded from http://mgltools.scripps.edu., Discovery Studio Visualizer 2019 was downloaded from http://www.3dsbiovia.com, Chemdraw Free Pro 8.0 was downloaded from http://chemistry.com.pk, Autodock Vina was downloaded from http://vina.scripps.edu (Narayanaswamy et al. 2016) and IBM SPSS Statistics was downloaded from https://www.ibm.com/analytics/spss-statistics-software.

IN VITRO XANTHINE OXIDASE INHIBITORY ASSAY

XO inhibitory assay was carried out using a modified method (Loh et al. 2021). Allopurinol was used as a positive control in the assay mixture. The reaction was performed in triplicates in a 96-well UV transparent microplate. The substrate and the enzyme solutions were prepared immediately before use. Test samples (apigenin, kaempferol and 4-hydroxybenzoic acid) were dissolved in 1% DMSO and diluted to 100 µg/mL. The assay mixture consisted of 130 µL of 0.05 M potassium phosphate buffer (pH 7.5), 10 µL of test sample and 10 µL of 0.1 unit/mL XO enzyme solution. After pre-incubation for 10 min at 25 °C, the reaction was initiated by the addition of 100 µL xanthine (0.15 mM) solution and incubated for 10 min at 30 °C. The enzymatic conversion of xanthine to form uric acid was measured at the absorbance of 295 nm. The XO inhibitory activity was calculated as:

$$\text{XOI activity (\%)} = [(\text{control absorbance} - \text{sample absorbance}) / \text{control absorbance}] \times 100$$

INTERACTION ASSAY

The XO inhibitory assay was repeated using different molar ratios of compounds (apigenin, kaempferol and 4-hydroxybenzoic acid). Test samples were dissolved in 1% DMSO and diluted to different concentrations (1 µM, 2 µM and 4 µM). Molar ratios of 1:1, 1:2, 2:1, 1:4 and 4:1 were chosen for this assay to investigate the interaction between [apigenin]:[kaempferol], [apigenin]:[4-hydroxybenzoic acid], and [kaempferol]:[4-hydroxybenzoic acid]. The reactions were performed in triplicates in a 96-well UV transparent microplate. *Va* and *Vb* are denoted as the relative enzyme activity in the presence of the two separate inhibitors, respectively. *Vab* is defined as the relative enzyme activity in the presence of the combination of the two respective inhibitors. If the inhibitory effect on XO of two respective inhibitors is independent, the expected relative remnant activity of XO, *Vc* (*Vc* = *Va* × *Vb*) is equal to *Vab*, and the combined effect is termed additive (*AD*). If the values of *Vab* - *Vc* below -0.10, the combined effect is considered as synergistic (*SY*) and the values above +0.10 were defined as antagonistic (AN) (Wang et al. 2015).

MOLECULAR DOCKING SIMULATION

The X-ray crystal structure of XO in complex with quercetin (3NVY) was downloaded from the RCSB Protein Data Bank, http://www.rcsb.org (Cao, Pauff & Hille 2014). The X-ray crystal structure of the XO was in complex with quercetin, a natural flavonoid inhibitor at 2.0 Å resolution. Before the protein was used for docking purposes, some molecules such as water molecules, cofactors and the left domain of the protein were removed using discovery studio visualizer. The protein was then saved in pdb format. The 3D structures of the apigenin, kaempferol and 4-hydroxybenzoic acid were generated using Chemdraw Free Pro 8.0 and all the structures were saved in pdb format. Quercetin and allopurinol were used as positive reference. The ligands and protein in pdb format were converted into pdbqt format using Autodock Tools-1.5.6 and docking calculation was performed using Autodock Vina. A total of 10 docked conformations were run as a result. To implement docking simulations, a grid box was defined with dimensions of center_x = 87.621, center_y = 7.785 and center_z = 16.029. For two ligands docking, the best scoring docked model of the primary ligand-XO complex was chosen to represent its most favorable binding and was used for the docking of a secondary ligand.

STATISTICAL ANALYSIS

The data was expressed as mean ± standard deviation (SD). Statistical analysis was performed using IBM SPSS software, Version 21. The data was analysed using one-way analysis of variance (ANOVA), and the significance of difference between the means was analysed by Duncan Post Hoc Tests, where $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

IN VITRO XANTHINE OXIDASE INHIBITORY ASSAY

In the present study, apigenin showed the highest XO inhibitory activity, which was not significantly different from the positive control, allopurinol (Figure 2). The inhibitory activity of apigenin was 30.3% and 39.4% higher than that of kaempferol and 4-hydroxybenzoic acid, respectively. Kaempferol and 4-hydroxybenzoic acid showed moderate inhibitory activity (High: 71-100%; moderate: 41-70%; low: 0-40%) on XO. This indicated that apigenin is a stronger XO inhibitor compared to kaempferol and 4-hydroxybenzoic acid. This agreed with the results reported by Cos et al. (1998), in which flavones showed slightly higher inhibitory activity than flavonols. Apigenin and kaempferol are flavone and flavonol, respectively. Umamaheswari et al. (2011)

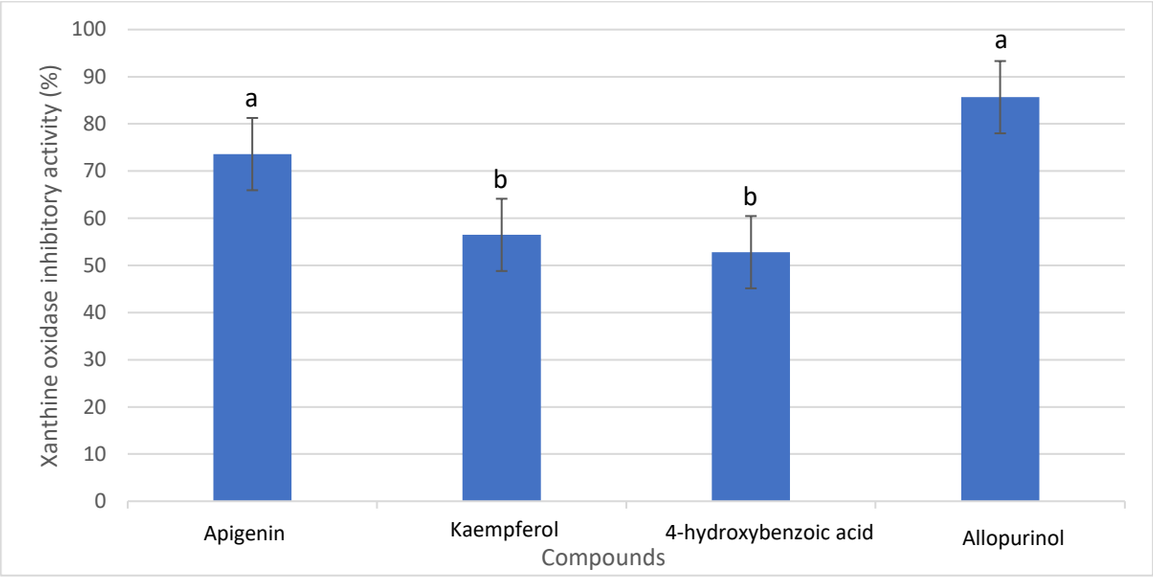

FIGURE 2. Xanthine oxidase inhibitory activity (mean ± SD) of apigenin, kaempferol and 4-hydroxybenzoic acid. Allopurinol serves as the positive control. Bars with different letters are significantly different ($p \leq 0.05$)

suggested that flavonoids are potential XO inhibitors due to their ability to interact with the active site of XO.

According to Masuda et al. (2013), phenolic acids are weaker XO inhibitor compared to flavonoids. Our finding also corroborated with the findings of Li et al. (2018) who indicated that 4-hydroxybenzoic acid possesses weak XO inhibitory potential. Phenolic acids are less affective in XO inhibition as they interact with the residues, not the active site of thrombin enzyme. Although kaempferol and 4-hydroxybenzoic acid showed only moderate inhibition towards XO, the combination of these compounds may enhance the efficiency.

INTERACTION ASSAY

Our results showed additive (AD) interactions between apigenin:kaempferol, apigenin:4-hydroxybenzoic acid (Table 1) and kaempferol:4-hydroxybenzoic acid (Table 2) at all molar ratios tested. According to Wang et al. (2015), additive effect refers to the combined inhibitory effect of two inhibitors (V_{ab}) equals to the sum of each inhibitor alone (V_c). Additive effects on the inhibition of XO have been shown between kaempferol and morin (or luteolin) (Wang et al. 2015) as well as between crysin and apigenin (Lin et al. 2015b) at different ratios and concentrations.

Independent apigenin and kaempferol are known to inhibit XO competitively by binding to the active site (Lin et al. 2015a). In the interaction assay, the additive interaction might indicate the binding of any two of the ligands simultaneously at the same active site at different dispositions. In other words, additive interaction indicates two independent ligands bound to the active site at non-interfering regional location (Lin et al. 2015b). In this study, the additive interaction between the compounds tested was explored further by using molecular docking simulation.

IN SILICO MOLECULAR DOCKING OF A SINGLE LIGAND TO XANTHINE OXIDASE

Molecular docking was used to improve the understanding of the interaction between ligands and the XO enzyme. Each of the ligand was docked with XO using Autodock Vina. A total of 10 different docked poses were generated and the binding energies were ranked accordingly. The binding energies of the 10 different docked poses were shown in Table 3. Allopurinol and quercetin were used as positive reference compounds. Apigenin demonstrated the greatest docking stability with XO due to its most negative binding energy of -8.7 kcal/mol among the compounds analysed. The binding energy refers to the energy released when the ligand bound to the protein. A more negative binding energy indicates greater stability for the ligand-enzyme complex formed (Du et al. 2016). The best-docked pose of the ligand-enzyme complex in 2D diagram was also shown in Figure 4. The 2D diagrams show the type of interactions involved in the binding between the ligand and the enzyme. According to Umamaheswari et al. (2011), hydrogen bonding and pi-pi (π-π) hydrophobic interactions held between the ligand and the amino acid residues of the enzyme are important in giving the biological activity, especially for potent anti-gout compound. The 2D diagram in Figure 4(a) shows that pi-pi hydrophobic interactions were formed between the B ring of apigenin and the phenylalanine of Phe914 and Phe1009. The hydroxyl groups of C7 and C4' also formed important hydrogen bonding with the amino acid residues, Asn768 and Arg880, respectively. According to Okamoto et al. (2008), Arg880 plays an important role in enzyme reaction by forming hydrogen bonding. Another hydrogen bond was found between the carbonyl group at C4 and amino acid residue Ser876. The orientation of apigenin demonstrated in this study was contrary to the findings by Lin et al. (2002), in which their study reported that the bicyclic benzopyranone ring of apigenin stacks with phenyl of Phe 914, and the phenolic group stretches to the space surrounded by several hydrophobic residues. The hydroxyl moiety at C7, C4' and the carbonyl group at C4 were shown to contribute favourable hydrogen bonds and electrostatic interactions with the active site, as shown in our results.

Figure 4(b) shows the 2D diagram displaying interactions between kaempferol and XO enzyme. The binding orientation of kaempferol was similar to apigenin, in which the B ring was sandwiched between amino acids Phe914 and Phe1009 via pi-pi hydrophobic interactions while the bicyclic benzopyranone stretched to the hydrophobic surrounding pocket. Two hydrogen bonds were also found between kaempferol and important amino acid residues, Ser876 and Asn768. On the other hand, the bicyclic rings of apigenin and kaempferol were inserted into the hydrophobic region of XO, interacting with residues Leu648, Pro1076, Leu873 and Val1011. This suggested that apigenin and kaempferol inhibits XO via insertion into the hydrophobic cavity of XO, occupying the active site of the enzyme. The present study agreed with Wang et al. (2015), who stated that the main inhibitory mechanism of kaempferol on XO activity is due to the insertion of kaempferol into the active site of XO, thereby preventing the entrance of the substrate and inducing conformational changes of XO.

TABLE 1. Interaction among apigenin and kaempferol (or 4-hydroxybenzoic acid) at different molar ratios

Compounds	Apigenin (1 μM)			Apigenin (2 μM)			Apigenin (4 μM)		
	Value		Interaction	Value		Interaction	Value		Interaction
	Obsd (*Vab*)	Exp (*Vc*)	*Vab - Vc*	Obsd (*Vab*)	Exp (*Vc*)	*Vab - Vc*	Obsd (*Vab*)	Exp (*Vc*)	*Vab - Vc*
Kaempferol (1μM)	5.77×10^{-3}	3.19×10^{-5}	5.73×10^{-3} (*AD*)	5.60×10^{-3}	7.45×10^{-5}	5.33×10^{-3} (*AD*)	4.83×10^{-3}	5.04×10^{-5}	4.78×10^{-3} (*AD*)
Kaempferol (2μM)	3.50×10^{-3}	3.68×10^{-5}	3.46×10^{-3} (*AD*)		-			-	
Kaempferol (4μM)	6.23×10^{-3}	2.36×10^{-5}	6.21×10^{-3} (*AD*)		-			-	
4-hydroxybenzoic acid (1μM)	1.40×10^{-2}	3.46×10^{-5}	5.73×10^{-3} (*AD*)	5.30×10^{-3}	8.10×10^{-5}	5.22×10^{-3} (*AD*)	9.63×10^{-3}	5.48×10^{-5}	9.58×10^{-3} (*AD*)
4-hydroxybenzoic acid (2μM)	5.73×10^{-3}	2.45×10^{-5}	5.71×10^{-3} (*AD*)		-			-	
4-hydroxybenzoic acid (4μM)	5.73×10^{-3}	3.96×10^{-5}	5.69×10^{-3} (*AD*)		-			-	

AD, additive interaction; Obsd, observed; Exp, expected; *Vab*, combined inhibitory effect of two inhibitors; *Vc*, the sum inhibitory effect of each inhibitor alone

TABLE 2. Interaction between kaempferol and 4-hydroxybenzoic acid at different molar ratios

Compounds	Kaempferol (1 μM)			Kaempferol (2 μM)			Kaempferol (4 μM)		
	Value		Interaction	Value		Interaction	Value		Interaction
	Obsd (*Vab*)	Exp (*Vc*)	*Vab - Vc*	Obsd (*Vab*)	Exp (*Vc*)	*Vab - Vc*	Obsd (*Vab*)	Exp (*Vc*)	*Vab - Vc*
4-hydroxybenzoic acid (1μM)	1.17×10^{-2}	5.70×10^{-5}	5.71×10^{-3} (*AD*)	4.00×10^{-3}	6.58×10^{-5}	3.90×10^{-3} (*AD*)	1.04×10^{-2}	4.22×10^{-5}	1.03×10^{-2} (*AD*)
4-hydroxybenzoic acid (2μM)	1.35×10^{-2}	4.03×10^{-5}	1.34×10^{-2} (*AD*)		-			-	
4-hydroxybenzoic acid (4μM)	2.47×10^{-3}	6.51×10^{-5}	2.40×10^{-3} (*AD*)		-			-	

AD, additive interaction; Obsd, observed; Exp, expected; *Vab*, combined inhibitory effect of two inhibitors; *Vc*, the sum inhibitory effect of each inhibitor alone

TABLE 3. The summary of binding energies for 10 binding modes obtained from the molecular docking of a single ligand to xanthine oxidase

Binding mode	Binding energy (kcal/mol)				
	Apigenin	Kaempferol	4-hydroxybenzoic acid	Allopurinol	Quercetin
1	-8.7	-7.7	-5.5	-5.2	-8.0
2	-8.3	-7.4	-5.4	-5.1	-7.6
3	-8.0	-7.3	-5.0	-5.1	-7.5
4	-7.8	-7.1	-4.9	-5.0	-7.3
5	-7.8	-7.1	-4.9	-5.0	-7.1
6	-6.7	-6.8	-4.9	-5.0	-6.9
7	-6.6	-6.8	-4.9	-4.9	-6.5
8	-6.4	-6.8	-4.8	-4.9	-6.4
9	-6.4	-6.7	-4.8	-4.8	-6.4
10	-6.3	-6.5	-4.7	-4.8	-6.3

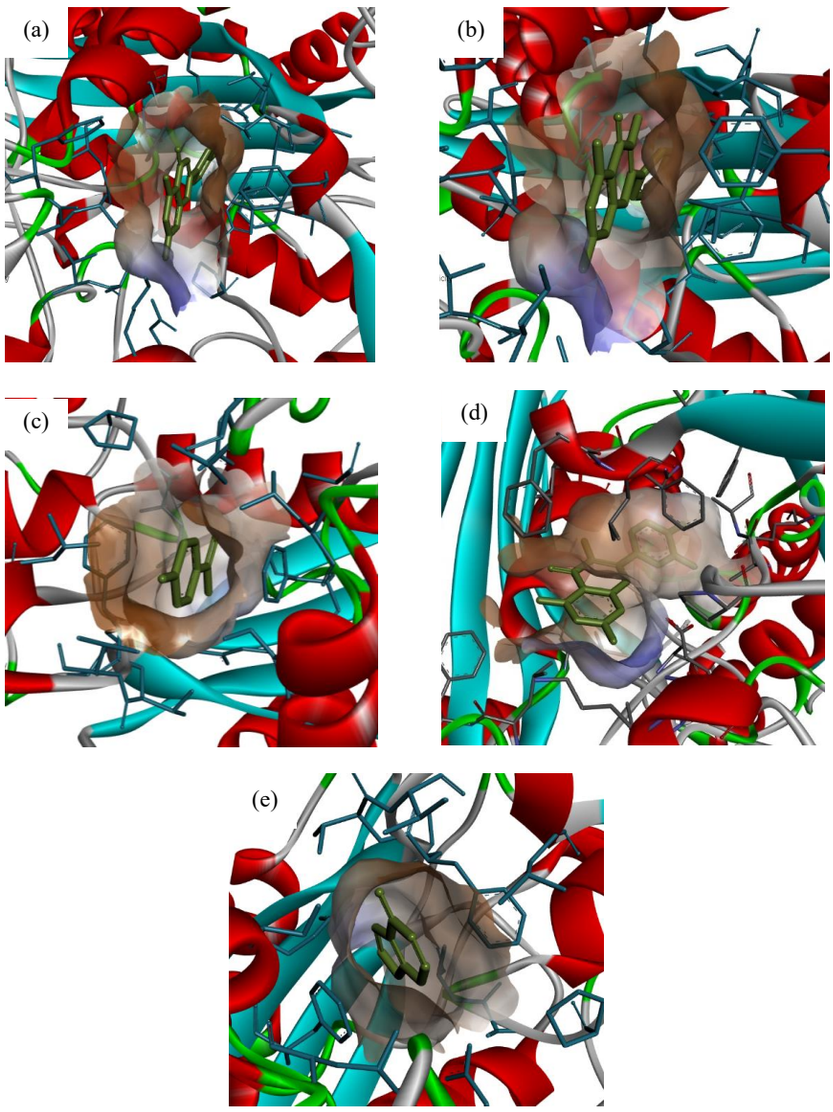

FIGURE 3. The best-docked pose of (a) apigenin, (b) kaempferol, (c) 4-hydroxybenzoic acid, (d) quercetin, and (e) allopurinol in the active site of xanthine oxidase enzyme

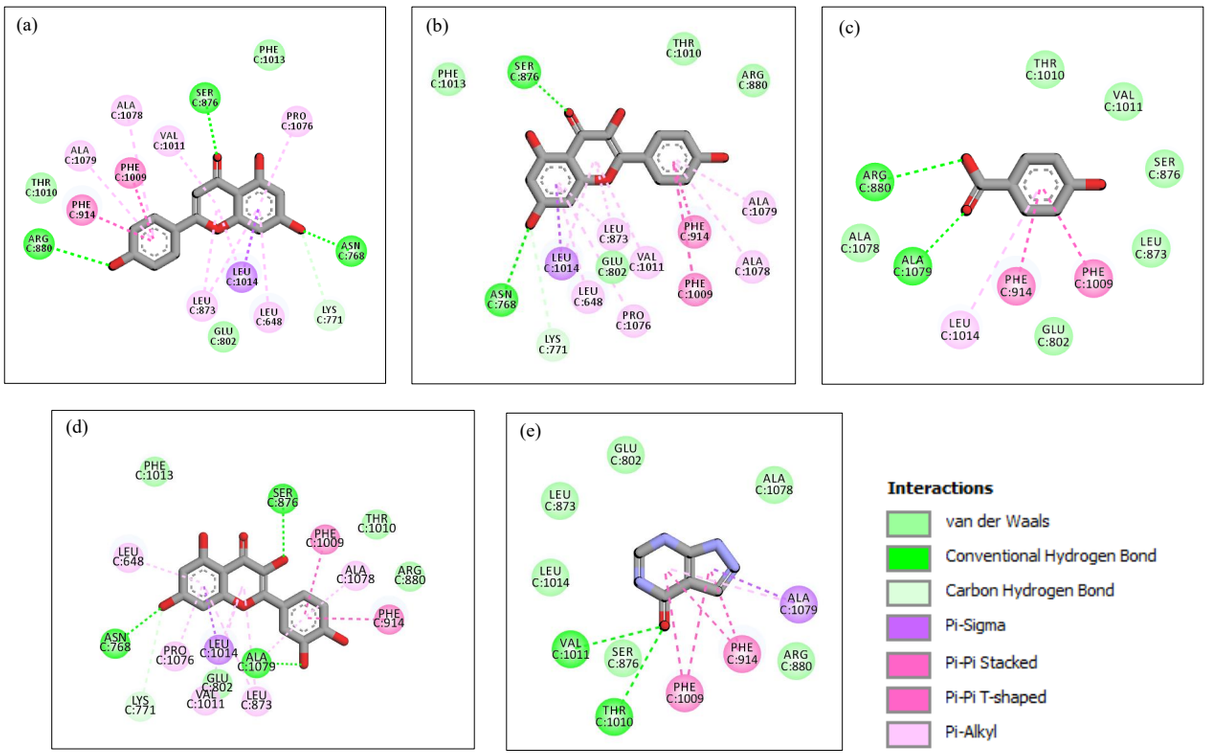

FIGURE 4. The type of interactions between the amino acid residues of xanthine oxidase enzyme and (a) apigenin, (b) kaempferol, (c) 4-hydroxybenzoic acid, (d) quercetin, and (e) allopurinol

Figure 4(c) shows pi-pi interactions between the benzene ring of 4-hydroxybenzoic acid and the Phe1009 and Phe914 amino acid residues of XO. Furthermore, 4-hydroxybenzoic acid formed hydrogen bonds with Arg880 and Ala1079 amino acid residues at the carboxyl group. On the other hand, 4-hydroxybenzoic acid also interacted with Thr1010, Val1011, Ser876, Leu873, Glu802, and Ala1078 via Van der Waals forces. This indicated that 4-hydroxybenzoic acid is also stabilised by both hydrophobic interactions and hydrogen bond in XO active site, similar to apigenin and kaempferol. Based on Tung and Chang (2010), other than the important residues Phe914, Phe1009, and Glu802, the other amino acid residues also contribute to the greater observed inhibitory activity of the ligands against XO.

Figure 4(d) shows the 2D diagram of the interactions between XO and quercetin (reference compound). The orientation of quercetin was similar to that of apigenin and kaempferol, in which the phenolic group formed pi-pi hydrophobic interactions with amino acid residues Phe914 and Phe1009. The bicyclic benzopyranone ring stretched to the hydrophobic pocket, forming hydrophobic interactions with Leu648, Pro1076, Leu873, and Val1011. This indicated that the mode of action of apigenin, kaempferol and quercetin is indistinguishable. Other than that, hydrogen bonds were formed between quercetin with Ser876, Asn768, and Ala1079 residues. Quercetin, apigenin, and kaempferol also formed Van der Waals interaction with Glu802, Thr1010, Phe1013 and Lys771 amino acid residues of XO. The lower binding energy in apigenin (-8.7 kcal/mol) and quercetin (-8.0 kcal/mol) might be attributed to the three hydrogen bonds that give additional stability to their interaction with XO enzyme compared to only two hydrogen bonds formed in kaempferol (-7.7 kcal/mol).

The *in vitro* study used allopurinol as a positive reference compound. Hence, the interaction between allopurinol and XO might give some insights about the important residues involved in the inhibition of the enzyme. The results showed that the bicyclic ring of allopurinol formed pi-pi hydrophobic interactions with Phe914 and Phe1009 residues (Figure 4(e)), and the

carbonyl oxygen interacted with Val1010 and Thr1010 via hydrogen bonding. This observation was similar to all other ligands tested in this study. The Phe1009 and Phe914 amino acids are the important amino acids in ligand-XO recognition (Tung & Chang 2010). Apart from that, allopurinol also formed Van der Waals interactions with amino acid residues Arg880 and Glu802, as demonstrated by the other ligands tested. According to Cao, Pauff and Hille (2010), the Arg880 and Glu802 residues in XO are important amino acids involved in converting xanthine into uric acid. The binding of the ligands to these two residues might halt the conversion, thereby inhibiting XO activity.

IN SILICO MOLECULAR DOCKING OF TWO LIGANDS TO XANTHINE OXIDASE

In this part of the study, two ligands were docked sequentially into the active site of XO to determine the interaction between them in the inhibition of XO. The summary of binding energies for ten different combinations of ligands docked with XO was shown in Table 4. The results showed that 4-hydroxybenzoic acid-apigenin (-7.4 kcal/mol) was the most stable ligands combination docked to XO. However, when the sequence was reversed in the apigenin-4-hydroxybenzoic acid combination, the binding energy became less stable (-5.7 kcal/mol). The same phenomenon was observed for other ligand pairs, such as the more stable kaempferol-apigenin (-6.5 kcal/mol) versus the less stable apigenin-kaempferol (-5.8 kcal/mol), and the more stable 4-hydroxybenzoic acid-kaempferol (-6.3 kcal/mol) versus the less stable kaempferol-4-hydroxybenzoic acid (-5.5 kcal/mol). This indicated that the sequence of docking the ligands into XO played an important role in affecting the stability of the multiple ligands-protein complexes. The possible factor that contributes to the stability of the secondary ligand might be the difference in the molecular size and the functional groups present in the primary (first-docked) ligand.

The most energetically favourable 3D binding pose of the multiple ligands-protein complexes was selected for binding orientation analysis (Figure 5). The 3D diagrams showed that the ligands docked independently at non-interfering region of the XO active site. Both ligands did not compete with each other for the same cavity, but their presence in the active site concurrently blocked the

TABLE 4. The summary of binding energies for the sequential docking of ligands to xanthine oxidase. The sequence of docking follows the order of compounds mentioned in each pair

Binding modes	Binding energy (kcal/mol)					
	PHBA- api	Api-PHBA	Kaemp-api	Api-kaemp	PHBA-kaemp	Kaemp-PHBA
1	-7.4	-5.7	-6.5	-5.8	-6.3	-5.5
2	-6.7	-5.5	-6.3	-5.7	-6.3	-5.4
3	-6.6	-5.4	-6.1	-5.6	-6.1	-5.2
4	-6.6	-5.2	-5.5	-5.5	-5.7	-5.0
5	-6.3	-5.2	-5.4	-5.2	-5.7	-4.8
6	-6.2	-4.9	-5.4	-5.1	-5.7	-4.6
7	-6.1	-4.9	-5.3	-5.1	-5.6	-4.6
8	-6.1	-4.6	-5.2	-4.9	-5.6	-4.5
9	-6.0	-4.5	-5.2	-4.8	-5.5	-4.2
10	-5.9	-4.4	-5.2	-4.8	-5.4	-4.1

The values indicate the binding energy of a secondary ligand to the primary ligand-protein complex. Api, apigenin; PHBA, 4-hydroxybenzoic acid; kaemp, kaempferol

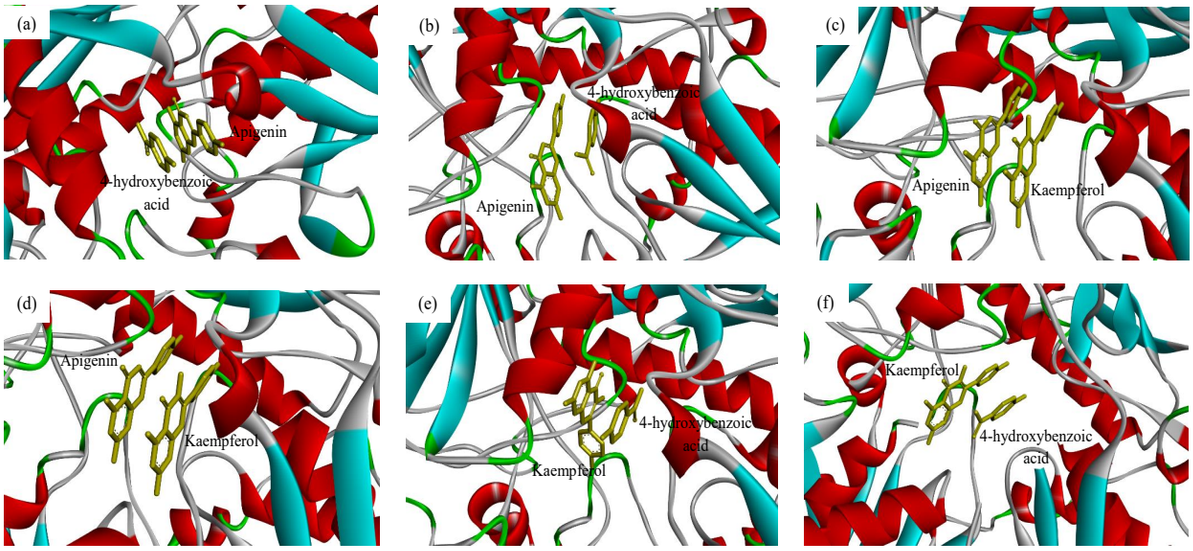

FIGURE 5. The best-docked pose of (a) 4-hydroxybenzoic acid-apigenin, (b) apigenin-4-hydroxybenzoic, (c) kaempferol-apigenin, (d) apigenin-kaempferol, (e) 4-hydroxybenzoic acid-kaempferol, and (f) kaempferol-4-hydroxybenzoic acid in the active site of xanthine oxidase enzyme. The sequence of docking follows the order of compounds mentioned in each pair

binding of xanthine substrate, thereby inhibiting XO activity. This suggested additive interaction by the combination of the ligands, in which the combined inhibitory effect of two ligands is equal to the sum of each ligand alone. The docking results further supported the additive interaction demonstrated in the *in vitro* XO inhibitory assay.

The 2D diagram shows the interactions involved in stabilising the secondary ligand to the primary ligand-protein complex. Figure 6 shows that when 4-hydroxybenzoic acid and apigenin were docked sequentially to XO, hydrogen bonds were formed between the Ser876 residue of XO and the C4 carbonyl and C5 hydroxyl of apigenin. The bicyclic benzopyranone ring of apigenin also formed hydrophobic pi-pi T-shaped interaction with Phe649. The binding was also stabilised by Pi sigma bond between A ring and B ring with Val1011 and Leu1014, respectively. However, when the ligands were docked in a reverse sequence, 4-hydroxybenzoic acid was only bonded to XO via a hydrogen bond between the carboxyl group and Ala1079 and a pi-anion interaction between the benzene ring and Glu1261 amino acid. This result conformed to the lower (more favourable) binding energy showed by the sequential docking of 4-hydroxybenzoic acid-apigenin compared to that of the apigenin-4-hydroxybenzoic acid docking.

Majority of the interactions between apigenin and kaempferol-XO complex were hydrophobic interactions (Figure 7(a)). A pi-pi T-shaped interaction was formed between the bicyclic benzopyran ring and Phe1013 amino acid. Other than that, pi-sigma interactions were formed between A and B ring of apigenin with Met770 and Leu1014 residues, respectively. Pi-sulfur interactions were also formed between Lys771 and Met770 with the phenolic group. However, an unfavourable acceptor-acceptor interaction was formed at the C7 hydroxyl group, which makes kaempferol-apigenin to be less favorable than 4-hydroxybenzoic acid-apigenin docking sequence in XO inhibition. This might be due to the small molecule size of 4-hydroxybenzoic acid (molecular weight of 138.12 g/mol) which allowed apigenin (molecular weight of 270.0528 g/mol) to fit better in the active site of the XO at non-interfering region. Reynolds, Bembenek and Tounge (2007) stated that ligand with smaller molecular size has greater ligand efficiency due to its advantage in terms of molecular properties. Therefore, the 4-hydroxybenzoic acid-XO

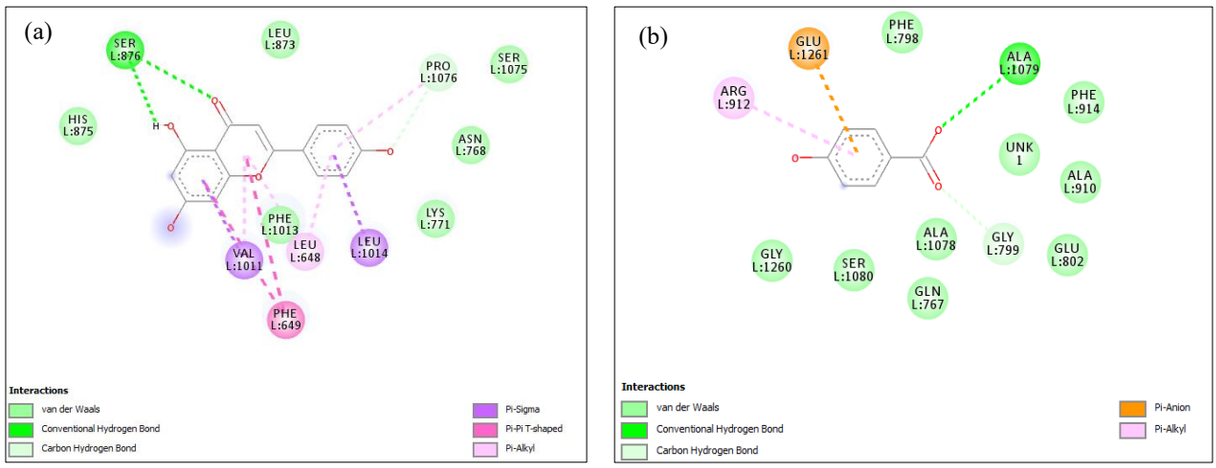

FIGURE 6. The type of interactions stabilising the docking of (a) apigenin to PHBA-XO complex in PHBA-api docking sequence, (b) 4-hydroxybenzoic acid to api-XO complex in api-PHBA docking sequence. PHBA, 4-hydroxybenzoic acid; api, apigenin

complex was speculated to be more stable and favoured the reception of the secondary ligand.

Nevertheless, less hydrophobic interaction was formed between kaempferol and apigenin-XO complex when the docking sequence was reversed (Figure 7(b)). Hydrogen bonds were formed between C5 hydroxyl with Glu879, C7 hydroxyl with Thr1010 and phenolic group with an unknown residue. Amino acid Glu879 also formed pi-anion interactions with the bicyclic benzopyran ring. The B ring of apigenin was found interacted with Val1011 via a pi-sigma bond. Since both hydrogen bonding and pi-pi (π-π) hydrophobic interactions are important in giving the biological activity of inhibitors (Umamaheswari et al. 2011), apigenin-kaempferol docking sequence might be less stable due to the lack of hydrophobic interactions.

The interactions involved between kaempferol and 4-hydroxybenzoic acid-XO complex are as shown in Figure 8(a). This docking sequence of ligands (4-hydroxybenzoic acid-kaempferol) showed slightly lower binding energy with XO than 4-hydroxybenzoic acid-apigenin. The small sized 4-hydroxybenzoic acid allowed important hydrogen bonds to be formed at C5 and C7 hydroxyl, and pi-pi stacked interactions formed between the bicyclic benzopyran ring and the B ring of kaempferol with Phe1013 and Phe649, respectively. The interaction was also stabilised by other hydrophobic interactions that involved amino acid residues Leu104, Pro1076, and Leu648. On the other hand, kaempferol-4-hydroxybenzoic acid resulted in a relatively unfavourable interaction with XO. As shown in Figure 8(b), 4-hydroxybenzoic acid was mainly stabilised by Van der Waals forces other than the hydrogen bond formed with Glu1261 and the hydrophobic pi interactions formed with Arg912 and Glu1261.

Our molecular docking results supported the *in vitro* results, in which all three combinations of ligand pairs demonstrated additive interaction in XO inhibition. However, the molecular recognition process in reality often involved more than one ligand rather than single ligand docked to the enzyme sequentially. This could be one of the drawbacks of our multiple ligands sequential docking simulation design. Hence, in order to simulate the real molecular docking processes, a multiple ligand simultaneous docking method is suggested to improve the docking simulation available.

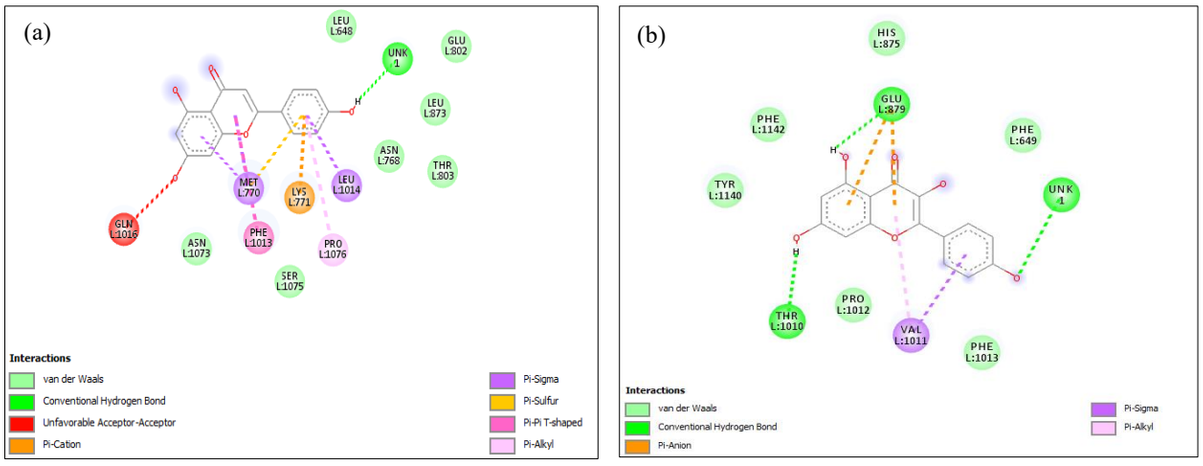

FIGURE 7. The type of interactions stabilising the docking of (a) apigenin to kaemp-XO complex in kaemp-api docking sequence, (b) kaempferol to api-XO complex in api-kaemp docking sequence. Kaemp, kaempferol; api, apigenin

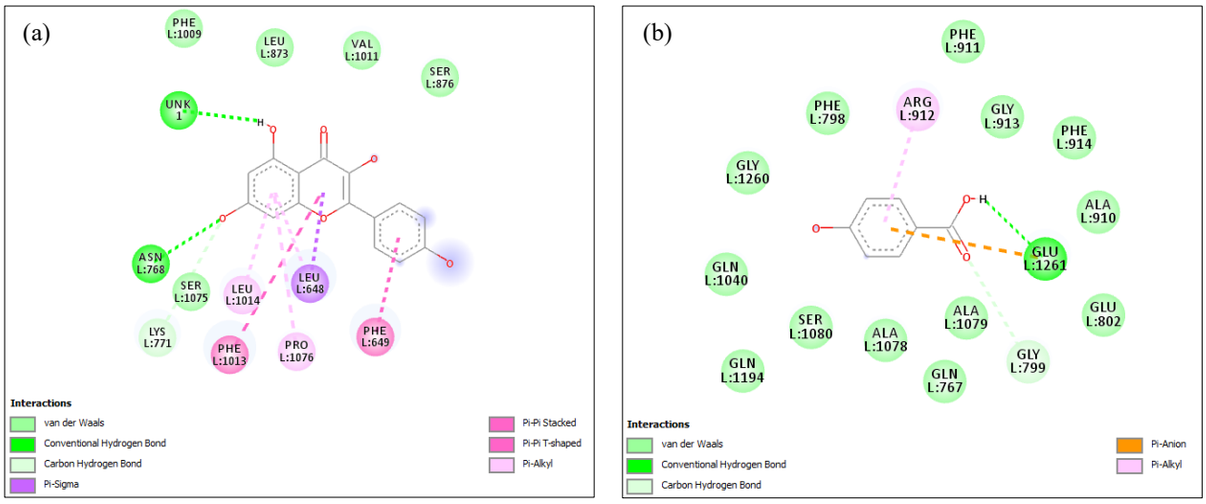

FIGURE 8. The type of interactions stabilising the docking of (a) kaempferol to PHBA-XO complex in PHBA-kaemp docking sequence, (b) 4-hydroxybenzoic acid to kaemp-XO complex in kaemp-PHBA docking sequence. PHBA, 4-hydroxybenzoic acid; kaemp, kaempferol

CONCLUSIONS

Apigenin was the most effective xanthine oxidase inhibitor compared to kaempferol and 4-hydroxybenzoic acid, which showed only moderate inhibition. The interaction between the three potential inhibitors were investigated using interaction assay where the results displayed additive interaction between apigenin-kaempferol, apigenin-4-hydroxybenzoic acid and 4-hydroxybenzoic acid-kaempferol at all molar ratios tested. The *in vitro* results were further supported when apigenin demonstrated the most favourable binding energy, followed by kaempferol and 4-hydroxybenzoic

acid in the *in silico* study. The interaction between the three ligands were also investigated further by using *in silico* multiple ligands docking simulation, in which additive interaction was also demonstrated when different combinations of two ligands were docked sequentially into the active site of XO. In a nutshell, apigenin, kaempferol, and 4-hydroxybenzoic acid can be potential inhibitors of xanthine oxidase enzyme in treating hyperuricemia related disease. The additive interaction between these compounds is suggested to be explored further for the enhanced effects and reduced adverse reactions when used in combination at lower doses.

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