Evaluation of Antioxidant Activity and α-Amylase Inhibitory Potential of *Melilotus indicus* Ethanolic Extract: An In Vitro and In Silico Study

Abstract:

Building upon the long history of plants as natural remedies, this study investigated the presence and content of flavonoids, a class of polyphenols, in an ethanolic extract of *Melilotus indicus*. The antioxidant activity of the extract was also evaluated. The Melilotus genus is renowned for its diverse biological activities, including antioxidant, anti-inflammatory, and hypoglycemic effects. As anticipated, flavonoids were the primary phenolic constituents identified in M. indicus. The extract exhibited a flavonoid content of 0.49 ± 3.818mg/g and demonstrated comparable antioxidant activity (IC50 = 1.6 mg/mL) to vitamin C (IC50 = 0.01 mg/mL). A docking study revealed promising binding scores for chlorogenic acid and kaempferol (-6.09196091 and -5.45953274 kcal/mol, respectively) compared to the acarbose drug (-8.8864727 kcal/mol). These findings suggest that M. indicus may be a valuable source of natural antioxidants with potential health benefits.

Keywords: *Melilotus indicus*, α-amylase, Chlorogenic acid, Kaempferol, Antioxidant

1. Introduction:

Medicinal plants have long played a pivotal role in human health, serving as both food sources and traditional remedies for various ailments. Moreover, they are considered promising sources for novel drug discovery and modification. *Melilotus indicus* is a medicinal plant that has been used both as a food and in traditional medicine. It is known for its analgesic and emollient properties (Abd El-Hafeez *et al*., 2018).This species belongs to the Fabaceae (Leguminosae) family and is rich in phytochemicals such as alkaloids, flavonoids such as quercetin, coumarins, triterpenes, and saponins(Ragab et al., 2021). Notably, it contains high levels of phenolic acids, particularly ferulic acid and chlorogenic acid, which contribute to its potent antioxidant activity, traditional medicine has employed Melilotus to treat a range of conditions, including asthma, hemorrhoids, bowel complaints, infantile diarrhea, and lacerated wounds(Ragab et al., 2022). In recent years, researchers have focused on exploring its potential anticancer properties due to its high flavonoid content(Tiwari & Mishra, 2017) .Flavonoids are a class of polyphenolic compounds found in plants, particularly fruits, vegetables, and beverages. They possess various beneficial biochemical and antioxidant effects, which have been linked to the prevention of diseases like cancer and Alzheimer's disease(Panche*et al*., 2016). Phenolic acids are another group of compounds found in plants, known for their antioxidant, anti-inflammatory, and antimicrobial properties. They also play a role in food preservation(Robbins, 2003). One of the most traditional uses of *Melilotus indicus* in Asia as anti-diabetic specifically as alpha-amylase inhibitor and this activity may interruption according the presence of many compounds such phenolic compounds(Ahmed *et al.*, 2014).

Given the abundance of *Melilotus indicus* in Iraq, it presents an excellent opportunity for further research into its biological activities. The variation in environmental conditions across different

regions can influence the composition of its active constituents, including coumarins, flavonoids, and phenolic acids. This diversity offers a rich source for exploring potential therapeutic applications.

2. Materials and methods:

2.1 Plant collection:

The plants arial parts were collected from the pharmacy college garden and were identified by Dr. Ula Almousawii as *Melilotus indicus*. Figure 1 illustrates the leaves and flowers of the plant, while Figure 2 shows the distribution of *Melilotus indicus* in Iraq. The aerial parts of the plant were dried in the shade until completely dry. The dried material weighed 15 grams and was used for the extraction process.

Figure 1: Leaves and Flowers of *Melilotus indicus*

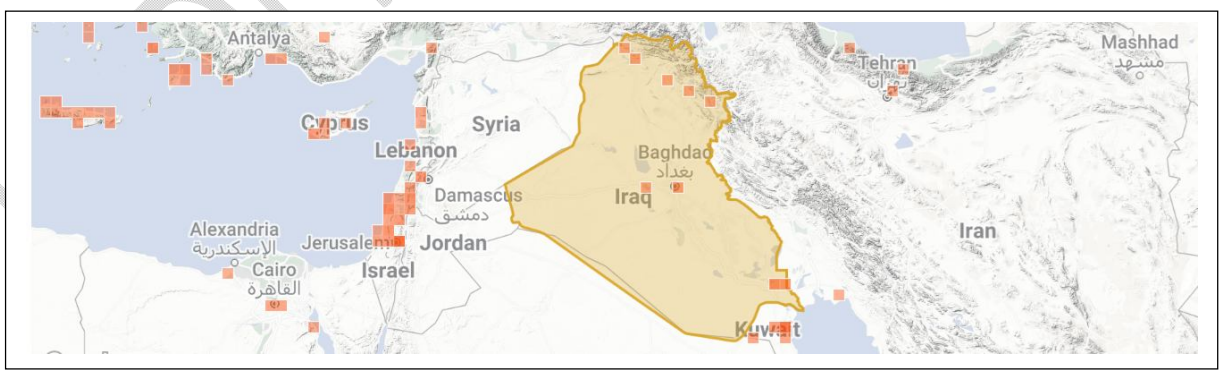


Figure 2: Iraqi map for *Melilotus indicus* distribution

2.2 Extraction]

The plant material was transferred to a round-bottom flask equipped with a reflux condenser. An appropriate amount of 80% ethanol solution was added to completely submerge the material, totaling 250 mL. The reflux apparatus was assembled, ensuring all connections were secure. The solution was heated

using a heating mantle to the boiling point of ethanol (approximately 70°C). The mixture was maintained under gentle reflux for 2 hours, allowing the condensed ethanol vapors to continuously return to the flask through the condenser. After refluxing, the solution was allowed to cool to room temperature. The cooled solution was then filtered using filter paper to separate the plant extract (liquid) from the plant residue. The filtrate was transferred to a clean Petri dish and left to dry at room temperature (Chaves *et al.*, 2020a).

2.3 Determination of flavonoids content

Total flavonoid content was determined by the aluminum chloride method. 0.5 ml of the ethanolic extract mixed with 0.3 ml of 5% sodium nitrite. After 5 min 0.3 ml of 10% aluminum chloride was added. After 6 min, we add 2.0 ml of 1 M sodium hydroxide and the total volume was made up to 5.0 ml with distilled water. The absorbance of the mixture was measured at 510 nm against a reagent blank. We use Quercetin as a standard. The flavonoid content was expressed as milligram of quercetin equivalence (QE) per gram of extract(Sasikumar, 2014).

2.4 Estimation of Melilotus Extract's Antioxidant action

2.4.1 Radical (DPPH) scavenging capacity

Melilotus indicus ethanolic extract antioxidant action was measured as a radical scavenging capacity of DPPH. The Erenler *et al.* (2016) method with a little modification was followed to accomplish the experiment. The absorbance readings at 517 nm were decreased with the reduction of the radicals. 1ml of 0.8 mM (DPPH) methanolic solution was added to 1 ml of each concentration of *Melilotus indicus* ethanolic extract (0.005-10) mg/ml. The tubes were enclosed tightly and set aside for (0.5) hours in the dark then at 517 nm the absorbance against blank samples was measured and compared to thecalibration curve of ascorbic acid. The test was accomplished in triplicate. The radical % inhibition was gained for the (DPPH) by the below equation:

Inhibition%= A^0-A/A^0 *100

where I = inhibition of DPPH (%), A0 = control sample

absorbance and A = tested sample absorbance after 0.5 hour. The scavenging activity plotted graph against different *melilotus indicus* extract concentrations can be used for determining the IC50 value, which can be defined as the total antioxidant essential to decrease 50% of the initial radical (Erenler et al., 2016), concentration. Reference compound was the Ascorbic acid (Labiad*et al*., 2017).

2.4.2 Statical analysis:

T-tests were performed to evaluate the results of the DPPH scavenging activity assay experiments.

2.5 Molecular Docking of some *Melilotus indicus* phenolic compounds into human alpha-amylase:

2.5.1 Medicinal compound choice

In this study, we aimed to investigate the potential of two phenolic acids (ferulic acid and chlorogenic acid), two flavonoids (quercetin and kaempferol), and coumarin (all found in *Melilotus indicus* aerial parts)(Ragab *et al*., 2022), as amylase inhibitors. These compounds were compared to the acarbose drug. An in-silico study was conducted using human salivary amylase (1C8Q), obtained from the Protein Data Bank (PDB).

2.5.2 Preparation of both enzyme and ligands

The three-dimensional structure of human salivary amylase was downloaded from the Protein Data Bank (PDB) using the PDB ID 1C8Q. The crystallographic properties of 1C8Q are summarized in Table 1. To identify the most suitable region of the receptor for ligand interactions, an active site prediction and isolation protocol was employed first The Hamiltonian PM3 (Parametric Model 3) method implemented in MOE was used to minimize the field strengths within the MMFF94x (Merck Molecular Force Field) energy of the protein. Additionally, water molecules were removed from the protein surface to ensure that the interaction region was not obscured. The active sites of 1C8Q were identified using the Site Finder model within MOE, as shown in Figure 3.

Table 1: Crystallographic properties of Human salivary alpha-amylase

Protein	PDB code	Classification	Organism	Expression system	Resolution	Method	Total structure weight (da)	Chain
Alpha-amylase	1C8Q	Hydrolase	Homo sapiens	*Spodoptera frugiperda*	2.30 A^0	X-ray diffraction	56030	A

Table 2 presents the chemical structures of the selected compounds and acarbose drug. The three-dimensional structures of these compounds were downloaded in SDF format from PubChem(Giménez *et al.*, 2010).Lipinski's physicochemical parameter rules [(Alanagreh*et al*., 2020), were also evaluated for each selected compounds (ligands) and the results are reported in Table 3. Also,the selected compounds were submitted to energyminimizing under default conditions of pH = 7 andtemperature = 300°K.

Table 2: The chemical structure of selected compoundsand acarbose drug

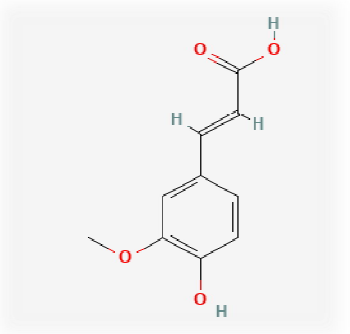
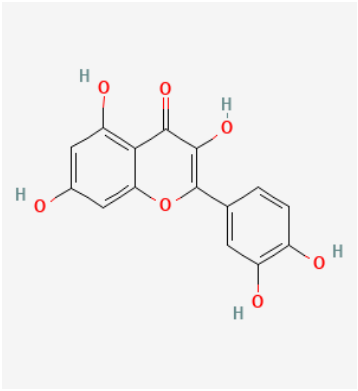
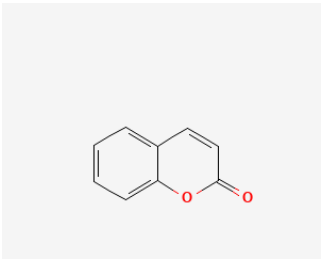
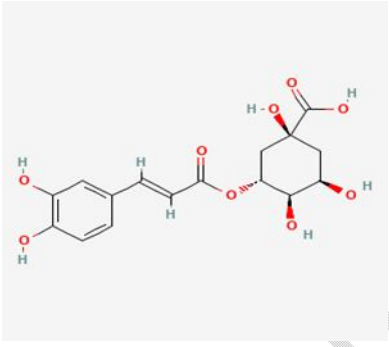
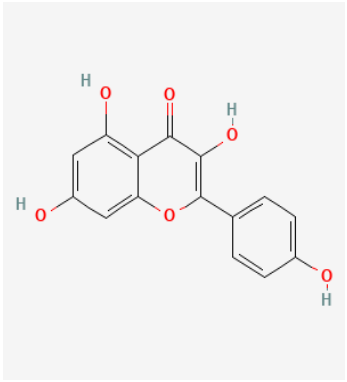
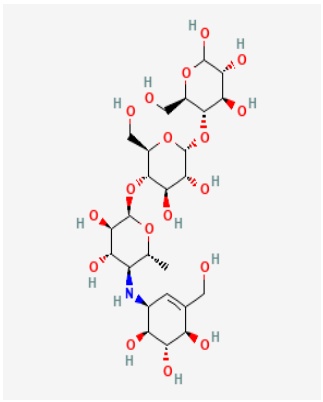
Phenolic acids	Flavonoids	Coumarin
 **Ferulic acid**		
		

Table 3: Physicochemical Lipinskis parameters for selected compounds and acarbose drug.

Compounds	MW gm/mol	h_log p	Lip_acc	Lip_don	Lip_druglike
Chlorogenic acid	354.311005	-0.280418485	9	6	1
Ferulic acid	194.185989	1.37839305	4	2	1
Quercetin	302.238007	1.75508523	7	5	1
Kaempferol	286.238983	2.23933625	6	4	1
Coumarin	146.144989	2.31558156	2	0	1
Acarbose	646.615967	-10.0566978	19	15	0

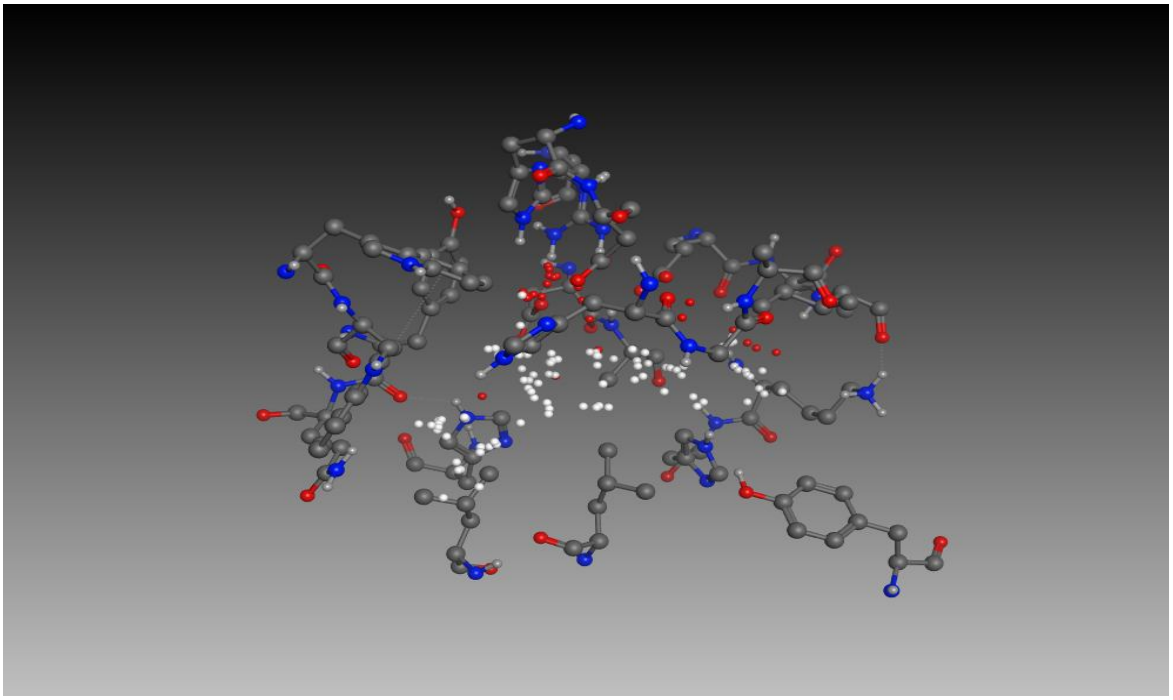


Figure 3: The active site of human salivary amylase (PDB ID 1C8Q), identified using MOE software.

2.5.3 Docking and Building Complexes

Docking was performed using the Dock module in MOE software, which involves positioning ligands within the active site of 1C8Q using most of the default settings to predict how molecules interact with the receptor's binding site(Arya *et al*., n.d.).The initial docked molecules included a series of compounds selected from *Melilotus indicus* and their respective reference inhibitors (acarbose drug, known as an amylase inhibitor). This allowed for a comparison of the obtained docking scores with those of the chosen ligands from the selected compounds.

3. Results and Discussion:

3.1 Extraction

The percentage yield of the dried extract was calculated using the following formula:

%Yield = (Weight of Dry Extract / Weight of Dry Plant Material) * 100%

% Yield = (11 g / 15 g) * 100% = 73.33%

3.2 Determination of total flavonoids content:

The total flavonoid content of the Melilotus ethanolic extract was determined to be 0.49 ± 3.818 mg/g. Aqueous ethanol is a well-established solvent for flavonoid extraction due to its ability to solubilize both aglycone and glycoside forms. Among aqueous alcohols, ethanol and methanol are generally preferred solvents for polyphenol compound extraction. Ethanol is often favored due to its lower toxicity.(Chaves *et al.*, 2020b).The selection of the reflux method with 80% ethanol for extraction aligns with the principle that water can enhance the efficiency of the extraction process. The presence of water in the solvent mixture facilitates the extraction of polyphenols by aiding their diffusion through plant tissues (Plaskova&Mlcek, 2023).

3.3 Antioxidant Activity

In this study, the antioxidant capacity of the Melilotus indicus ethanolic extract was evaluated. The IC50 value for the extract was determined to be 1.6 mg/mL, compared to 0.01 mg/mL for vitamin C. The p-value for this comparison was 0.188. Presence of flavonoids such as quercetin and kaempferol may lead to the antioxidant activity of *Melilotus indicus* because these compounds contain a hydroxyl group, The best-described antioxidant property offlavonoids derives from its ability to directly scavengethe reactive oxygen species, flavonoids can chelate freeradicals immediately by donating a hydrogen atom or bysingle-electron transfer, another possible mechanism ofaction of flavonoids is through the chelation of transitionmetal elements, flavonoids have chelating property,which enabled them to chelate, or binds to metal ions inthe human body to prevent them from being accessiblefor oxidation, flavonoids can also act as an intracellularantioxidant through inhibition of free radical generatingenzymes(Banjarnahor&Artanti, 2014).

3.4 Docking study

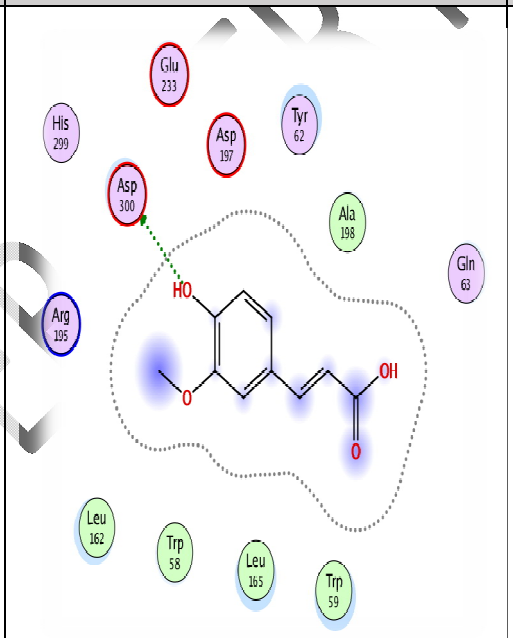
Table 4 summarizes the docking scores obtained for all selected compounds from Melilotus indicus aerial parts and the acarbose drug.

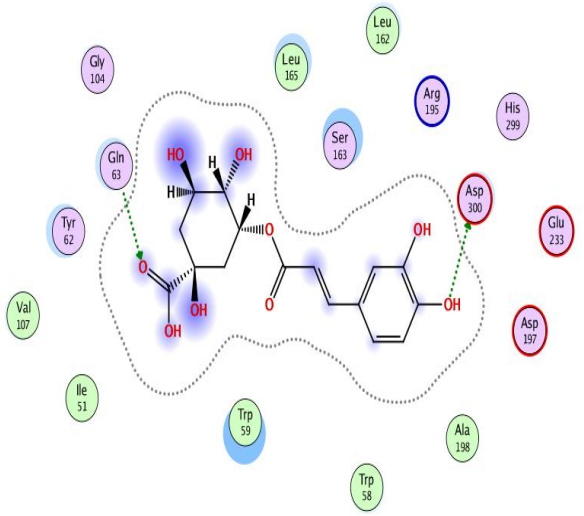
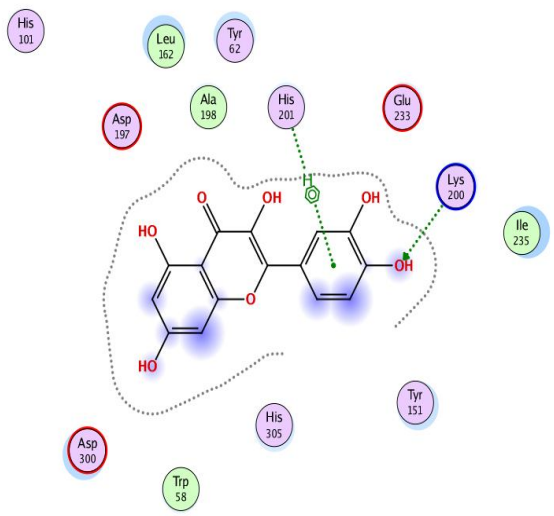
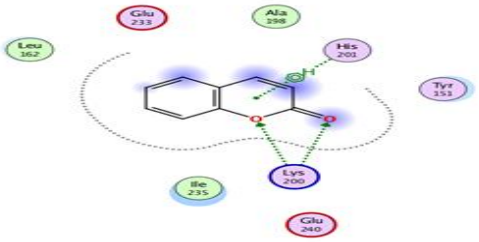
Table 4: The binding scores for the docking of 1C8Q with the selected compounds from *Melilotus indicus* aerial parts and acarbose

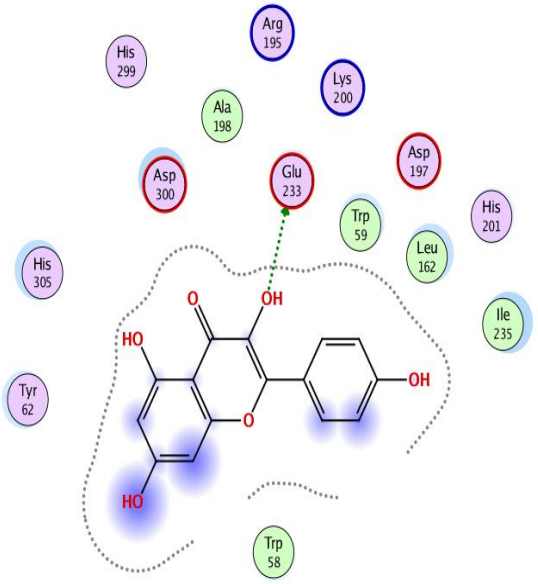
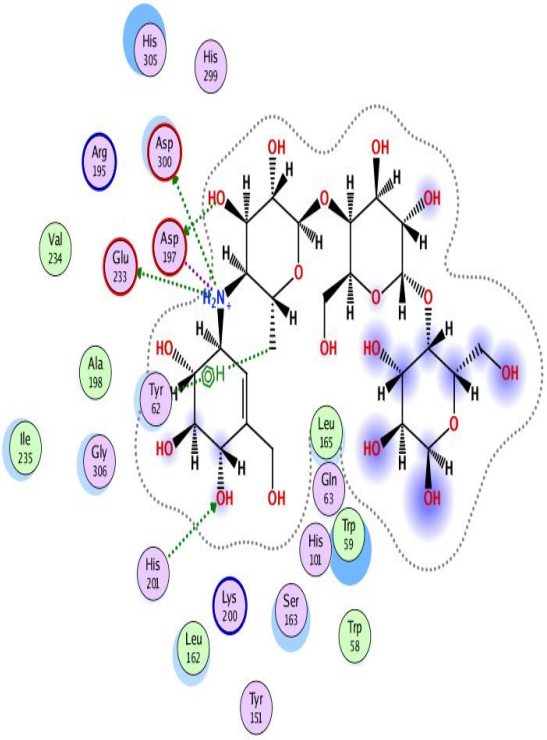
Selected compounds and acarbose	Binding score (kcal/mol) with 1C8Q
Ferulic acid	-4.62238932
Chlorogenic acid	-6.09196091
Quercetin	-5.2621212
Kaempferol	-5.45953274
Coumarin	-4.53506327
Acarbose	-8.8864727

The most favorable energy complex was formed by chlorogenic acid, with a binding energy of -6.09196091 kcal/mol, followed by kaempferol at -5.45953274 kcal/mol. In comparison, the acarbose-enzyme complex had a binding energy of -8.8864727 kcal/mol. Table 5 presents the in-silico binding complexes formed by docking the selected compounds with human salivary amylase, compared to the acarbose drug.

Table 5: illustrates the theoretical interactions produced by docking the selected compounds and acarbose drug with 1C8Q.

Selected compounds and drug	2D interaction	Types of binds
Ferulic acid		**The amino acid ASP 300 (H-donor) with 2.89 A^0 distance and energy of -5.0 kcal/mol**

Chlorogenic acid		**The amino acid ASP 300 (H-donor) with 2.96 A0 distance and energy of -4.8 kcal/mol** **The amino acid GLN 63 (H-acceptor) with 3.01 A^0 distance and energy of -1.1 kcal/mol**
Quercetin		**The amino acid LyS 200 (H-acceptor) with 3.02 A^0 distance and energy of -3.8 kcal/mol** **The amino acid HIS 201 (pi-H) with 3.89 A^0 distance and energy of -0.9 kcal/mol**
Coumarin		**LYS 200 (H-acceptor) with 3.17 A^0 distance with energy -1.1kcal/mol** **LYS 200 (H-acceptor) with 3.08 A^0 distance and energy of -5.0** **HIS 201 (pi-H) with 3.92 A^0 distance and energy -0.7kcal/mol** **GLU 233 (H-donor) with**

Kaempferol		**2.93 A^0 distance and energy of -3.7 kcal/mol**
Acarbose		**ASP 197 (H-donor) with 2.87 A^{0}distance and energy of -1.5 kcal/mol** **ASP 197 (H-donor) with 2.96A^{0}distance energy of -1.9 kcal/mol** **GLU 233 (H-donor) with 3.37 A^0 distance and energy of -4.7kcal/mol** **ASP 300 (H-donor) with 3.68 A^0 and energy of -1.1kcal/mol** **HIS 201 (H-acceptor) with 3.39 A^0 distance and energy of -0.8kcal/mol** **ASP 197 (Ionic) with 3.51 A^{0}distance and energy of -1.9kcal/mol** **GLU 233 (Ionic) with 3.37 A^{0}distance and energy of -2.4 kcal/mol** **GLU 233 (Ionic) with 3.28 A^{0}distance and energy of -2.9kcal/mol** **ASP 300 (Ionic) with 3.68 A^0 distance and energy of -1.3kcal/mol** **TYR 62 (H-pi) with 3.75 A^0 and energy of -0.6kcal/mol**

Salivary amylase is a glucose-polymer cleavage enzyme secreted by the salivary glands. It digests starch into smaller molecules, ultimately producing maltose. Maltase then cleaves maltose into two glucose molecules. This demonstrates the significant physiological role of saliva in food digestion. (Peyrot des Gachons& Breslin, 2016) . Numerous molecules exhibit α-amylase inhibitory activity, including flavonoids, phenolic acids, tannins, and terpenes. As previously mentioned, we selected flavonoids, phenolic acids, and coumarin for our docking study due to their established importance as α-amylase inhibitors.(da Silva et al., 2014).In Asia, *Melilotus indicus* has been traditionally used as an anti-diabetic agent due to its active constituents. Compared to acarbose, a pseudotetrasaccharide, Melilotus indicus has gained particular attention as a highly effective inhibitor of intestinal α-glucosidases and α-amylase. Acarbose is clinically used to treat both non-insulin-dependent and insulin-dependent diabetes mellitus, effectively lowering postprandial glucose elevation in diabetics. However, acarbose, like other drugs, can have side effects, including moderate diarrhea associated with flatulence. This often leads to therapy discontinuation, emphasizing the need for the development of new α-amylase inhibitors. (da Silva et al., 2014) . The potential side effects associated with synthetic α-amylase drug inhibitors have led many individuals to explore natural product alternatives derived from medicinal plants, such as *Melilotus indicus.*(Ahmed et al., 2014).Despite the promising docking scores obtained for chlorogenic acid, acarbose demonstrated the best results. While Melilotus indicus may offer potential therapeutic benefits, it is important to note that it may not be a direct replacement for drugs. This finding suggests the need for further research to modify chlorogenic acid into a more potent drug with reduced side effects and enhanced α-amylase inhibitory activity.

Conclusion:

This study concludes that Melilotus indicus contains a significant amount of flavonoids and exhibits potent antioxidant activity. The docking study revealed promising binding scores for chlorogenic acid and kaempferol.

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