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Exploring the potential of natural feed additives from herbs as an alternative to antibiotic growth promoters for Mojosari layer duck (*Anas javanica*) farming: *in-silico* and *in-vivo* studies

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ABSTRACT

Using synthetic feed additive, such as antibiotic growth promoters (AGPs), is common in Mojosari layer duck (*Anas javanica*) farms. Their purpose is to enhance growth and productivity. However, the indiscriminate use of AGPs has been associated with the emergence of bacterial resistance. This leads to lingering effects and potential health hazards for poultry and consumers. In light of these concerns, natural herb feed additives have emerged as an alternative strategy to AGPs in poultry feed. The active compounds in natural feed additives can improve feed efficiency and enhance production performance, resulting in higher-quality poultry products, especially eggs. However, it is known that the active compounds in phytobiotics derived from herbs contain anti-nutrients that can affect productivity and alpha-amylase in poultry. This study was aimed to investigate the interconnection between active compounds in phytobiotics and alpha-amylase using in-silico methods and verify the findings with field data using *in-vivo* methods. Alpha-Amylase is a vital enzyme that catalyzes the hydrolysis of the glycosidic bonds present in glycogen, resulting in the formation of maltose, a water-soluble disaccharide. Based on the results of docking involving nine phytobiotic compounds and their interaction with glycogen, it was observed that andrographidine E showed a binding affinity of 8.8 kcal/mol. Furthermore, these compounds form conventional hydrogen bonds, facilitated the formation of stable bonds between proteins and compounds. In particular, the interacting amino acids did not exhibit unfavourable bonds. Therefore, andrographidine E was more stable compared to other compounds. Nonetheless, the results show that several compounds have weaker binding affinity than the enzyme's binding to glycogen. However, the hydrophobic binding types of these compounds did not seem to impact the performance of the alpha-amylase enzyme, as demonstrated by *in vivo* data.

Keywords: Alpha-amylase, Anas javanica, Andrographidine E, Antibiotic growth promoters, Phytobiotic compounds

INTRODUCTION

The use of synthetic feed additives, such as antibiotic growth promoters (AGPs), are widespread in Mojosari layer duck farming to enhance growth and productivity. Mojosari layer ducks (*Anas javanica*), native Indonesian egg-laying ducks bred for high productivity, generally use AGPs. However, the indiscriminate use of AGPs has been associated with developing bacterial resistance, leading to residual effects and posing potential health risks to poultry and consumers (Untari *et al*., 2021). In light of these concerns, using natural feed additives derived from herbs has emerged as an alternative approach to AGPs in poultry feed. The exploration of the potential of herbs and spices has revealed active compounds that serve as natural feed additives, such as *Curcuma longa* (curcumin), *Zingiber Officinale* (gingerol), *Kaempferia galangal* (epigoitrin), *Andrographis paniculata* (andrographidine-e, 14-deoxy-11,12-didehydroandrographiside), *Tinospora crispa* (tyramine), *Phyllanthus niruri* (cianidanol, rutin) and *Piper betle Linn* (cyranoside). These natural feed additives have been shown to possess various beneficial properties, including antibacterial, anti-inflammatory, and antioxidant activities. Therefore, they hold great promise as a safer and more sustainable alternative to replace AGPs in producing Mojosari layer ducks. By adopting this approach, we can promote the production of healthier poultry products while reducing the potential risks associated with AGP use.

The active compounds in natural feed additives can increase feed efficiency and improve production performance, leading to better quality poultry products, especially eggs. However, it is known that active compounds in phytobiotics derived from herbs contain anti-nutrients that can affect productivity and alpha-amylase in poultry. Alpha-amylase is an enzyme derived from salivary glands that break down the grains' starch into monosaccharides by increasing starch digestibility (Seo *et al*., 2016). This makes it more efficient to increase egg production. This study aims to investigate the interconnection between active compounds in phytobiotics and alpha-amylase using *in-silico* methods and verify the findings with field data using in-vivo methods. The *in-silico* data included protein quality, secondary structure, interaction, phytobiotic compound analysis, and molecular docking. The egg production data obtained included both external and internal egg quality. External egg quality included egg weight, eggshell weight, egg index, shell thickness, and air cell size, while internal egg quality includes white and yolk index, haugh unit, yolk colour index, white and yolk volume, and yolk cholesterol. The combination of in-silico and in-vivo methods in this study was expected to provide a deeper understanding of active compounds' function and potential roles in herbs and spices, enabling their use to maximize poultry productivity in producing high-quality eggs for consumption.

MATERIALS AND METHODS

In vivo (Animal Trials and Experimental Diets)

This research began with the process of natural feed additive conducted in the Laboratory of Animal Feed Industry, Faculty of Animal Science, Brawijaya University. It started with an oven drying process at a temperature of 60°C for 3x24 hours until the moisture content was below 14%. After the natural feed additive was dried, it was further processed through grinding to obtain a powdered texture. The natural feed additive was filtered using nanoparticle principles, employing a micro-sized 100-mesh sieve, resulting in a powdered form that was used as a feed additive.

Field research was conducted for 60 days at a layer duck farm located on Sawahan Street, Turen, Malang Regency. The study involved 120 female Mojosari layer ducks (*Anas javanica*) aged 24 weeks (168 days) and was carried out in 24 postal cages. The environmental temperature in the cages ranged from 28 to 30°C, with humidity between 60% and 70%. The maintenance

equipment included feeders, drinkers, hygrometers, digital scales, and hanging scales. The equipment used to measure external and internal egg quality consisted of a digital scale with a capacity of 500 g and an accuracy of 0.1 g for weighing eggs and shells, egg trays, calipers, and micrometer screws. The research method employed a Completely Randomized Design (CRD). Six treatments were applied, with each treatment replicated four times, resulting in a total of 24 experimental units. Each experimental unit contained 5 female Mojosari layer ducks. The treatments were as follows: P0 (basal feed without antibiotics), P1 (basal feed + zinc bacitracin 0.1%), P2 (basal feed + 0.25% natural feed additive), P3 (basal feed + 0.5% natural feed additive), P4 (basal feed + 0.75% natural feed additive), and P5 (basal feed + 1% natural feed additive). Feed was provided restrictively in the morning at 07:00 and in the evening at 15:00, while water was provided *ad libitum* but controlled.

The measurement of research variables was conducted daily. The research variables consisted of egg weight, egg index, shell weight, shell thickness, air cell size, egg white index, haugh unit, egg white and yolk volume, yolk color, and yolk cholesterol test. Egg weight and shell weight were measured using an analytical balance. The egg index was measured by comparing the width and length diameters of the egg. Shell thickness was measured using a micrometer screw in three different parts: blunt end, middle, and pointed end (Prasetya *et al*., 2015). Measurement of air cell depth was done by taking a fragment from the blunt end of the egg. Then, the depth of the air cell was measured from the inner membrane that separated from the outer membrane of the eggshell using a caliper. The egg white index was measured by comparing the height of thick egg white divided by the average of the length and short diameters of the egg white. The yolk index was measured by comparing the yolk height with the egg diameter. Haugh unit calculation was derived from the measurement of albumin height and egg weight. Egg white and yolk volumes were measured using a measuring cup. Yolk color was measured by comparing the color of the yolk using the Roche Egg Yolk Colour Fan, which has a standardized color scale from 1 to 15. The yolk cholesterol test was conducted using the Liebermann Burchard method.

Statistical Analysis

The research data was recorded and tabulated using Excel software. Data was analyzed using analyse of variance from completely randomized design. If there is a significant difference between treatments, then continued by Duncan's multiple range test.

In Silico

The methodology employed in this study involved conducting *in silico* research using data mining techniques. The control ligand data and active compounds were obtained from the PubChem database, with the PubChem ID for the control being loureirin B (189670). The herbal waste was comprised of gingerol (442793), tyramine (5610), rutin (5280805), 14-deoxy-11,12-didehydroandrographiside (44575271), andrographidine-e (13963769), cianidanol (9064), cyranoside (5280637), epigoitrin (3032313), and curcumin (969516) (Wibowo *et al*, 2020). Furthermore, the study also involved obtaining data for protein modelling from Uniprot, with the code F1NW02 being used for this purpose. The research systematically gathered relevant data from reliable sources to support their *in silico* research. This methodology allowed us to analyze the molecular interactions and binding affinities between the ligands and target proteins, providing valuable insights into potential drug candidates for further research.

Protein Modelling: Protein modelling was performed using SWISS-MODEL by inputting the protein's amino acid sequence in FASTA format. The model was selected based on coverage, GMQE, QSGE, and identity from the template protein results. After successful protein modelling, the assessment process was performed using Procheck to determine the

protein's quality based on the Ramachandran Plot (Wibowo *et al*., 2020).

Protein secondary structure analysis: The tool ProtParam is utilized to calculate input protein sequences' physicochemical properties. These sequences can be in the format of Fasta or ID and SwissProt/TrEMBL accession number. ProtParam is an integrated tool with SwissProt available on web.expasy.org. The input parameters include molecular weight, theoretical pI, amino acid composition, atomic composition, and instability index (Wibowo *et al*., 2020).

Protein interaction: STITCH is an online tool that predicts protein interactions based on chemical structure similarities. It retrieves information from various databases and generates a network diagram of the protein's interactions with other proteins and chemicals, which can aid in understanding biological processes and diseases. The server also provides visualization tools for identifying key molecules and pathways (Khun *et al*., 2013).

Binding site analysis: P2Rank is a software tool for predicting protein-ligand binding sites. It analyses protein structure and uses machine learning algorithms to identify regions likely to interact with ligands. To use P2Rank, the user uploads a protein structure file in PDB format and runs the program, which generates a list of predicted binding sites ranked by a confidence score. The user can then analyze and visualize the predicted binding sites using various tools provided by P2Rank (Krivak & David, 2018; Wibowo *et al*., 2020).

Chemicals Analysis: SWISS ADME is an online tool that helps researchers analyze small molecules' pharmacokinetic and physicochemical properties in drug discovery and development. This tool offers the ability to evaluate potential drug efficacy, safety, and likeness. Uploading a chemical structure in SMILES, SDF, or MOL file formats to SWISS ADME allows for the calculation of various properties, including Lipinski's Rule of Five, which predicts bioavailability and absorption, solubility, toxicity, and metabolism-related parameters. Moreover, SWISS ADME also offers a 3D representation of the compound's structure and a list of related compounds in the database (Daina *et al*., 2017; Wibowo *et al*., 2022).

Molecular Docking: Protein preparation was done using Discovery Studio 2016 Client. The protein's water molecules and ligands were removed. The ligands were then prepared using Open Babel to convert (.sdf) to (.pdbqt) (Wibowo *et al*., 2019). Next, docking was performed using Autodock Vina, which was integrated with YASARA. The selected docking results ranked first (Wibowo *et al*., 2021). Analysis and visualization of docking data were done using Discovery Studio 2016 Client.

RESULTS AND DISCUSSION

External and Internal Egg Quality of Mojosari Laying Ducks

The data from the analysis of variance on the effect of treatments on the external egg quality of Mojosari laying ducks, included egg weight, shell weight, egg index, shell thickness,

Table 1. Data of External Egg Quality Caused by the Addition of Phytobiotic

Treatments	Variables				
	Egg Weight (gram)	Shell Weight (gram)	Egg Index (%)	Shell Thickness (mm)	Air Cell Size (cm)
P0	61.31± 2.80^{a}	8.10±0.36^{a}	77.83±0.70	0.54±0.03	1.52±0.09
P1	61.56±1.10^{ab}	8.07±0.13^{a}	76.73±0.69	0.53±0.01	1.52±0.06
P2	64.34±1.74^{ab}	8.46±0.16^{b}	76.43±1.38	0.56±0.03	1.53±0.10
P3	66.06±1.16^{bc}	8.65±0.09^{bc}	77.01±2.86	0.57±0.01	1.56±0.12
P4	67.75±1.65^{c}	8.84±0.18^{cd}	78.21±1.09	0.57±0.03	1.55±0.13
P5	71.50±0.76 ^{d}	9.18±0.12^{d}	77.35±2.14	0.57±0.02	1.56±0.01

Lowercase superscript letters in the same column indicate significant differences (P<0.01).

Table 2. Results of the Study on the Effect of Phytobiotic Addition on the Internal Quality of Mojosari Layer Duck Eggs

Treatments	Variables						
	Albumen index (mm)	Yolk index (mm)	Haugh Unit	Yolk Color	Albumen volume (ml)	Yolk volume (ml)	Yolk Cholesterol (mg/g)
P0	0.27±0.02	0.35±0.01	106.06±0.97	13.69±0.07^{a}	32.50±1.58^{ab}	20.31±2.08^{ab}	150.24±28.19
P1	0.28±0.02	0.36±0.02	106.70±1.23	13.72±0.19^{a}	31.81±1.16^{a}	21.06±0.33^{a}	174.42±24.80
P2	0.29±0.02	0.6±0.01	107.38±1.17	14.19±0.22^{b}	33.06±0.53^{ab}	23.88±0.34^{ab}	151.24±9.70
P3	0.29±0.01	0.36±0.01	108.04±0.84	14.28±0.21^{b}	34.16±0.99^{bc}	24.50±0.44^{bc}	160.58±10.67
P4	0.29±0.03	0.36±0.01	108.28±1.48	14.13±0.27^{b}	34.97±0.53^{c}	25.28±1.47^{c}	150.82±9.13
P5	0.27±0.02	0.36±0.01	108.50±1.20	14.50±0.10^{b}	36.78±0.74^{d}	26.53±2.18^{d}	148.38±11.40

Lowercase superscript letters in the same column indicate significant differences (P<0.01).

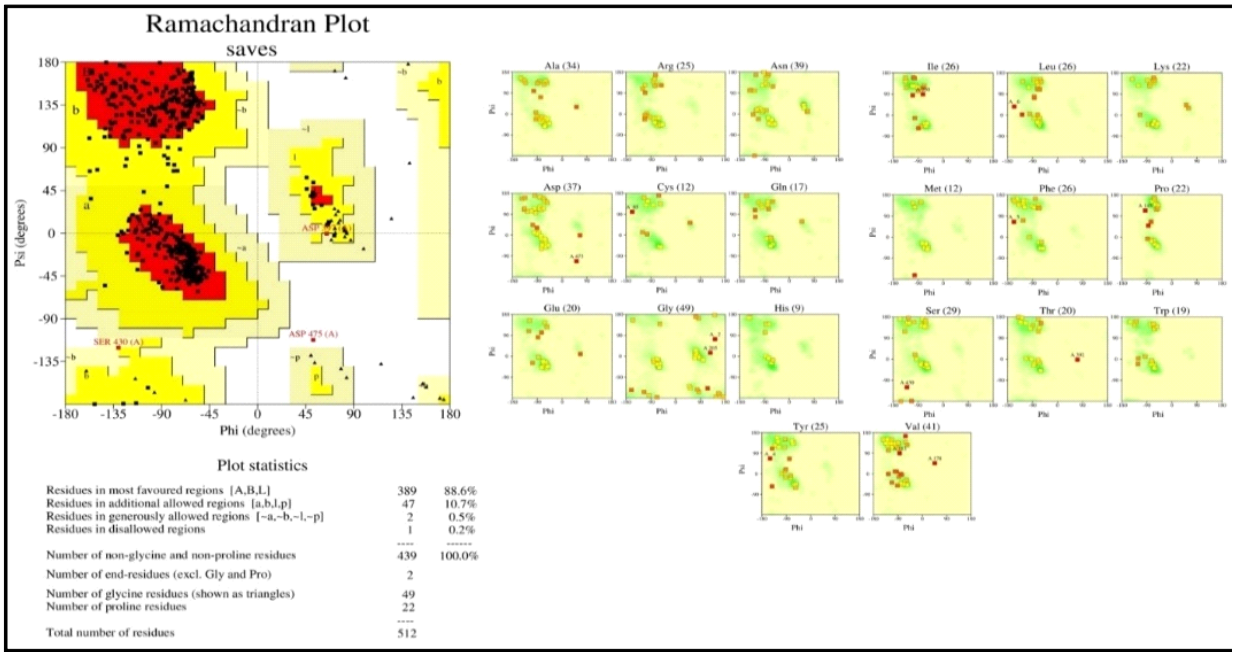

Figure 1. Protein Assessment of Alpha-amylase

and air cell size are presented in Table 1. The research results on adding phytobiotics up to a dose of 1% in feed on the weight of eggs and eggshells of Mojosari laying ducks showed significant differences (P<0.01) between the treatments. Adding up to a dose of 1% on egg index, eggshell thickness, and air cell showed no significant differences (P>0.05).

The highest egg weight product was 71.50 g, with an eggshell weight of 9.18 g. Carbohydrates, proteins, especially amino acids, fats, water, vitamins, and minerals, are a group of nutrients that play an important role in the egg formation process. Nutrient absorption from the feed in the digestive tract will be optimal if the digestive tract works properly, which is certainly separate from the role of active compounds in phytobiotics. This condition is certainly beneficial for poultry, which impacts increasing feed consumption. Nutrient absorption can be maximized so that an increase in egg production and egg quality can be achieved (Fawaz *et al*.,

Figure 2. Protein Structure Analysis of Alpha-amylase using ProtParam

2022).

Data about the effect of treatments on the internal quality of Mojosari laying duck eggs, included white egg index, yolk index, haugh unit, egg white and yolk volume, yolk colour, and yolk cholesterol are presented in Table 2. Eggs are the most complete chemical substance that approaches the chemical composition of the animal concerned. Most of the egg composition consists of water and other essential elements such as carbohydrates, proteins, fats, etc. The improvement of egg quality is achieved by adding feed additives such as phytobiotics to the diet.

Based on the Table 2, it was found that the addition of phytobiotics in the feed up to a dosage of 1% increased the yellow yolk colour index and the white and yellow yolk volume. However, it did not affect the variables of white and yellow yolk index, haugh unit, and yellow yolk cholesterol. To enhance the performance of poultry production, the role of alpha-amylase in breaking down the cell walls of feed particles to easily digested by poultry, leading to increase nutrient availability, cannot be overlooked (Aderibigbe *et al*., 2020).

It has an important role to increase ducks' egg weight and yolk diameter. Alpha-amylase is a crucial enzyme that catalyzes the hydrolysis of glycosidic bonds in glycogen, forming maltose, a water-soluble disaccharide. Data from the *in-silico* method to determine the interaction of phytobiotic active compounds in alpha-amylase consisting of protein assessment, protein interaction, binding site analysis, chemical analysis, and molecular docking can be seen in the Figure 1.

Protein Assessment

The protein structure model using the SWISS-MODEL server and subsequently evaluated using the Procheck web server, a program that validates protein modelling by analyzing Ramachandran plots. Ramachandran plots are two-dimensional plots that depict the amino acid residues in the internal coordinates of the experimentally determined enzyme structure. The quality assessment is based on the percentage of amino acid residues in the Ramachandran plot's most favoured regions and disallowed regions. The percentage of residues in the most favoured regions (A, B, L) was 88.6%, while the residues in additional allowed regions (a, b, l, p) accounted for 10.7%. The generously

allowed regions (-a, -b, -l, -p) had a percentage of 0.5%, and the residues in the disallowed regions were 0.2%. This percentage value indicates that the greater the number of protein residues in the most favoured regions, the higher the protein quality, making this protein valid for later stages of research. This approach demonstrates the efficacy of computational methods for protein structure modelling and quality assessment (Wibowo *et al*., 2020). Top of Form

Protein Structure Analysis

The protein structure (Figure 2) was analyzed using ProtParam, an integrated tool with SwissProt available on web.expasy.org. Proteins are composed of amino acid components linked and differ in their side chains (R groups). Based on the analysis, the alpha-amylase protein has a molecular weight of 57790.92 Daltons, a theoretical isoelectric point (pI) of 5.47, and is composed of 512 amino acids, included 34 alanines (6.6%), 25 arginines (4.9%), 39 asparagines (7.6%), 37 aspartic acids (7.2%), 12 cysteines (2.3%), 17 glutamines (3.3%), 20 glutamic acids (3.9%), 49 glycines (9.6%), 9 histidines (1.8%), 26 isoleucines (5.1%), 27 leucines (5.3%), 22 lysines (4.3%), 13 methionines (2.5%), 26 phenylalanines (5.1%), 22 prolines (4.3%), 29 serines (5.7%), 20 threonines (3.9%), 19 tryptophans (3.7%), 25 tyrosines (4.9%), and 41 valines (8.0%). The extinction coefficient is a characteristic that determines how strongly alpha amylase absorbs or reflects radiation or light at certain wavelengths depending on the structural composition of atoms, chemistry, and proteins (Ubi *et al*., 2022). This extinction coefficient at 280 nm is measured in water. The estimated-half life of protein is essential for estimating the half-life of proteins in tissues and cells (Rahman & Sadygov, 2017). The N-terminal of the sequence considered is M (Met). The estimated half-life is 30 hours for mammalian reticulocytes in vitro. More than 20 hours for yeast, *in vivo* and 10 hours for *Escherichia coli*, *in vivo*. The instability index is the method for predicting protein stability *in vivo*, depending on the structure of the main protein available. If the index is less than 40, it indicates a stable protein; if it is larger, it indicates an unstable protein (Gamage *et al*., 2019). The instability index (II) was 28.20, and the aliphatic index was 70.23. The aliphatic index plays a role in the thermal

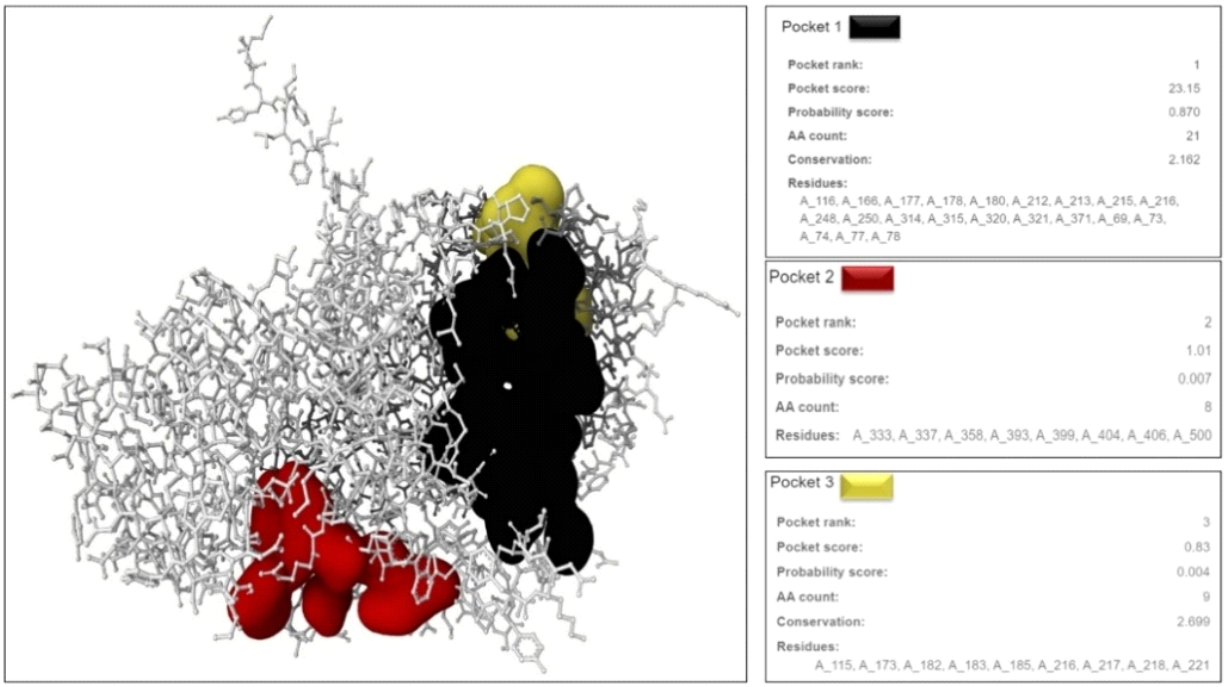

Figure 3. Binding Site Analysis of alpha-amylase

stability of proteins, with a range of 66.5 to 84.33, indicating that the protein is stable under thermal conditions and contains high amounts of hydrophobic amino acids (Panda & Chandra, 2012). Furthermore, the grand average of hydropathy (GRAVY) was -0.359, indicating that the alpha-amylase protein is a hydrophilic compound that can bind to water. In birds, alpha-amylase is secreted by salivary glands.

Binding Site Analysis

The pocket 1 is composed of atoms in residues A_116, A_166, A_177, A_180, A_212, A_213, A_215, A_216, A_248, A_250, A_314, A_315, A_320, A_321, A_371, A_69, A_73, A_74, A_77, A_78 in each chain. The pocket 2 comprises atoms in residues A_333, A_337, A_358, A_393, A_399, A_404, A_406, and A_500 in each chain. Pocket 3 is composed of atoms in residues A_115, A_173, A_182, A_183, A_216, A_217, A_218 and A_221 in each chain. The binding site analysis process involves identifying protein targets that exhibit activity in interacting with ligands. The analysis results reveal a high probability of alpha-amylase for specific ligand binding.

Protein Interaction (alpha-amylase with glycogen as control compounds)

Utilizing computational analysis, the results indicate that the protein alpha-amylases interaction within avian organisms facilitates the breakdown of complex carbohydrates, specifically glycogen, into polysaccharides, glucose, and maltose, thereby enabling facile digestion and absorption by the avian digestive tract.

Phytobiotics Compound Analysis

The physicochemical properties of phytobiotics compound candidates can be used to understand and predict their physiological absorption and determine possible biological effects after oral ingestion. These phytobiotics compounds can be calculated theoretically to improve and help create guidelines for understanding the behaviour of phytobiotics compound candidates in biological environments such as a gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeant, P-glycoprotein (P-gp) substrate, CYP1A2 inhibitor, CYP2C19 inhibitor, CYP2C9 inhibitor, CYP2D6 inhibitor, CYP3A4 inhibitor, and water solubility (Velez *et al*., 2022).

A comparison of GI predictions in these 9 phytochemicals shows that tyramine, curcumin, gingerol, epigoitrin, and cianidanol have the highest GI absorption. The permeability of phytobiotic compounds to the BBB determines their absorption in the brain; tyramine and gingerol show the highest absorption. The P-gp substrate is expressed in the intestinal epithelium and decreases the cellular uptake of its substrates (Velez *et al*., 2022). The results show that cianidanol, cynaroside, andrographidine-e, 14-deoxy-11,12-didehydroandrographiside, and rutin have the highest P-gp substrate. Cytochrome P450 (CYP) is a group of proteins responsible for the phase I metabolism of phytobiotic compounds (Bhatt *et al*., 2022). CYP1A2, an important isoform of CYP mainly expressed in the liver, is strongly inhibited by gingerols (Figure 5). CYP2C9 is vital in metabolizing anti-inflammatory drugs and biotransforms 12.8% of phytobiotics in the liver. Curcumin showed the most energetically favourable binding in docking to CYP2C9. CYP2D6 has an affinity for basic substrates, comprises 2% of the CYP content of hepatocytes, and is responsible for the biotransformation of 20% of phytobiotics undergoing hepatic metabolism. Gingerol was found to be a potent CYP2D6 inhibitor. CYP3A4 is the most prevalent and clinically significant cytochrome, responsible for the biotransformation of 30.2% of phytobiotics metabolized by the liver (Bhatt *et al*., 2022). Further examination of curcumin and andrographidine E potential as CYP3A4 inhibitors revealed that the active compound components within the phytobiotic can be readily absorbed into water solutions, rendering them amenable to docking procedures.

Molecular Docking

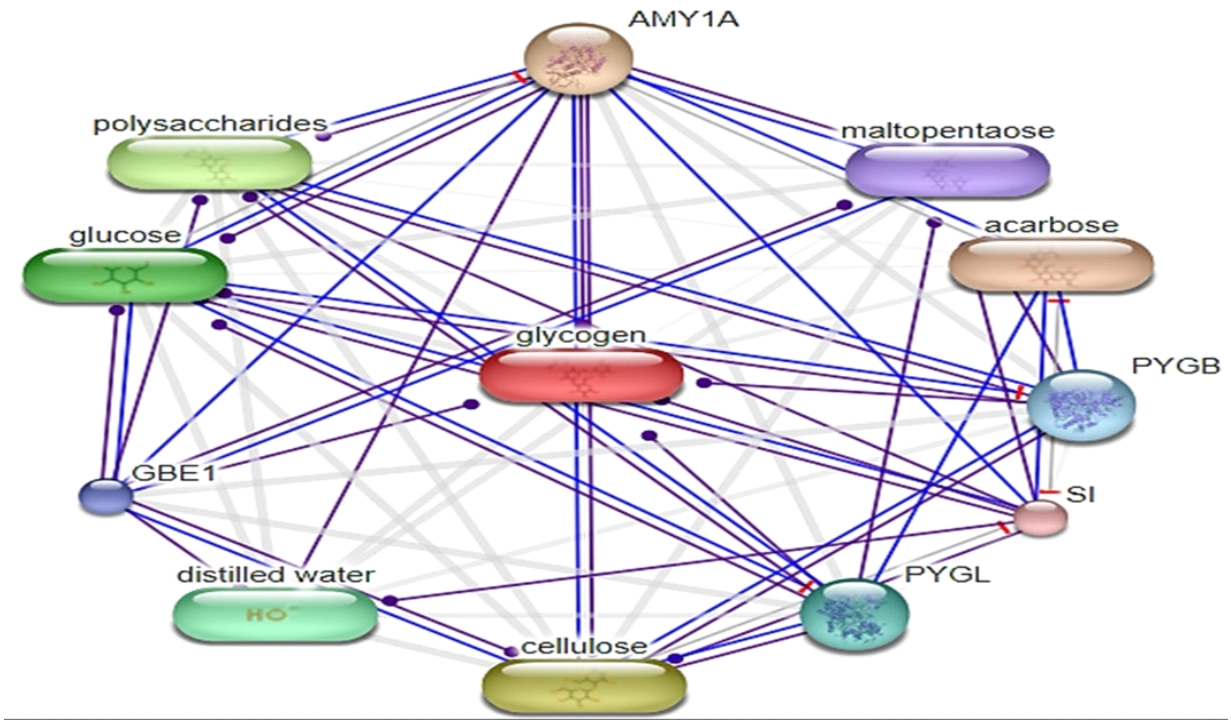

Figure 4. Protein Interaction

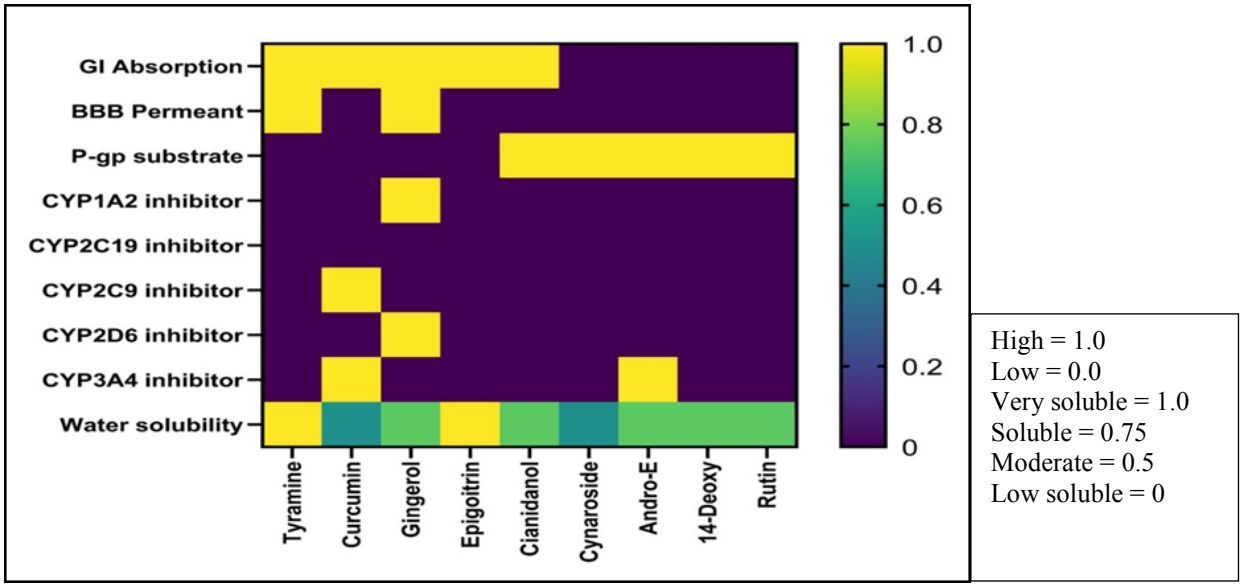

Figure 5. Phytobiotics Compound Analysis

The glycogen complex possesses a variety of bond types, including conventional hydrogen bonds with amino acids ASP(A):315, TYR (A):166, TRP(A):74, GLU(A):248, and GLY (A):321. van der Waals bonds are present with amino acids ALA(A):213, ARG(A):210, HIS (A):314, ASN(A):313, PHE(A):271, TRP(A):73, LEU(A):180, GLN(A):78, LEU(A):177, ILE (A):250, TYR(A):77, HIS(A):120, VAL(A):178, and GLY(A):319. Additionally, carbon-hydrogen bonds are present in amino acids HIS (2):216 and ASP(A):212.

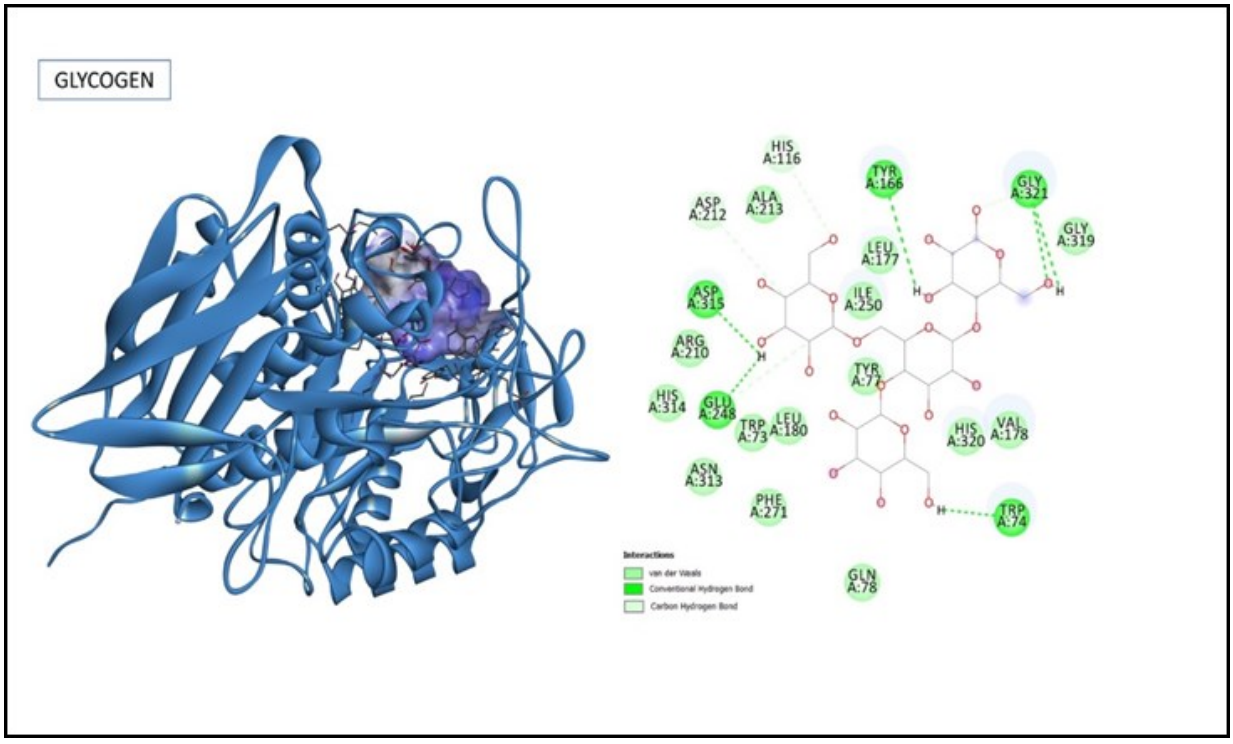

Figure 6. Glycogen (Control)

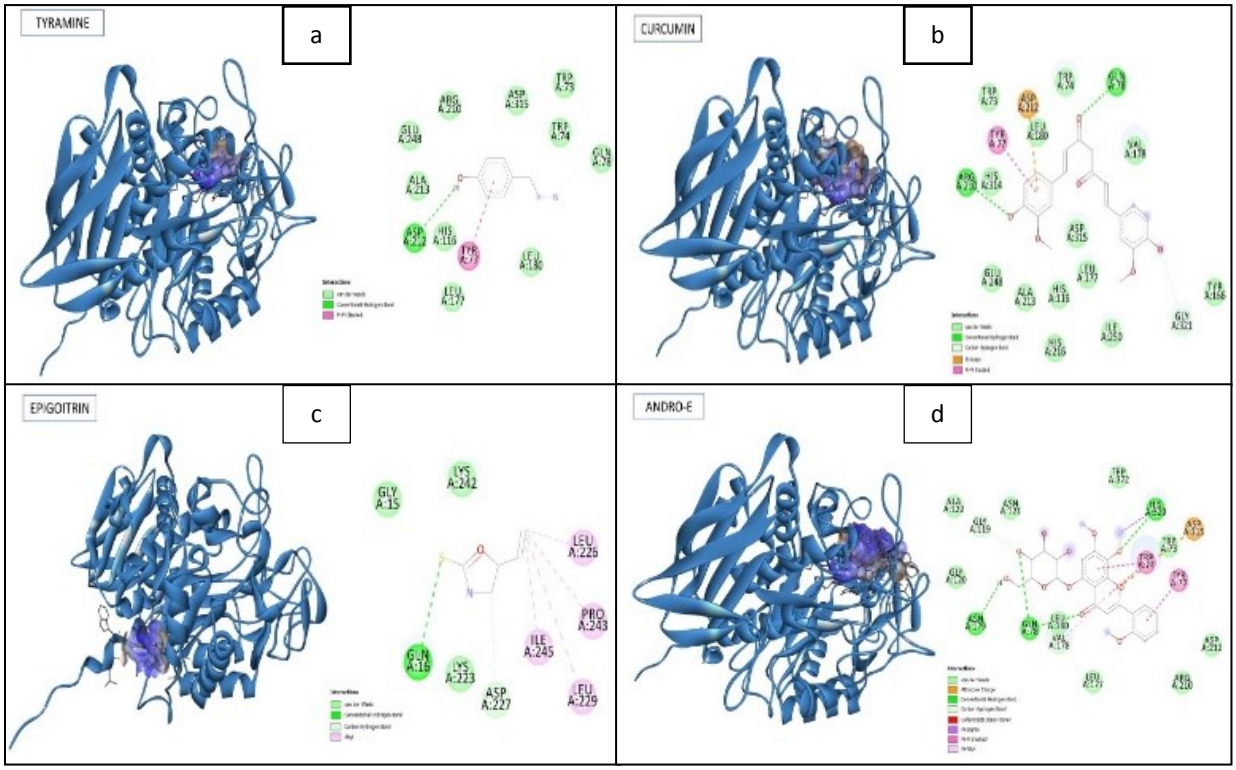

Figure 7. Visualization docking a. Tyramine b. Curcumin, c. Epigotrin, d. Andrographidine E

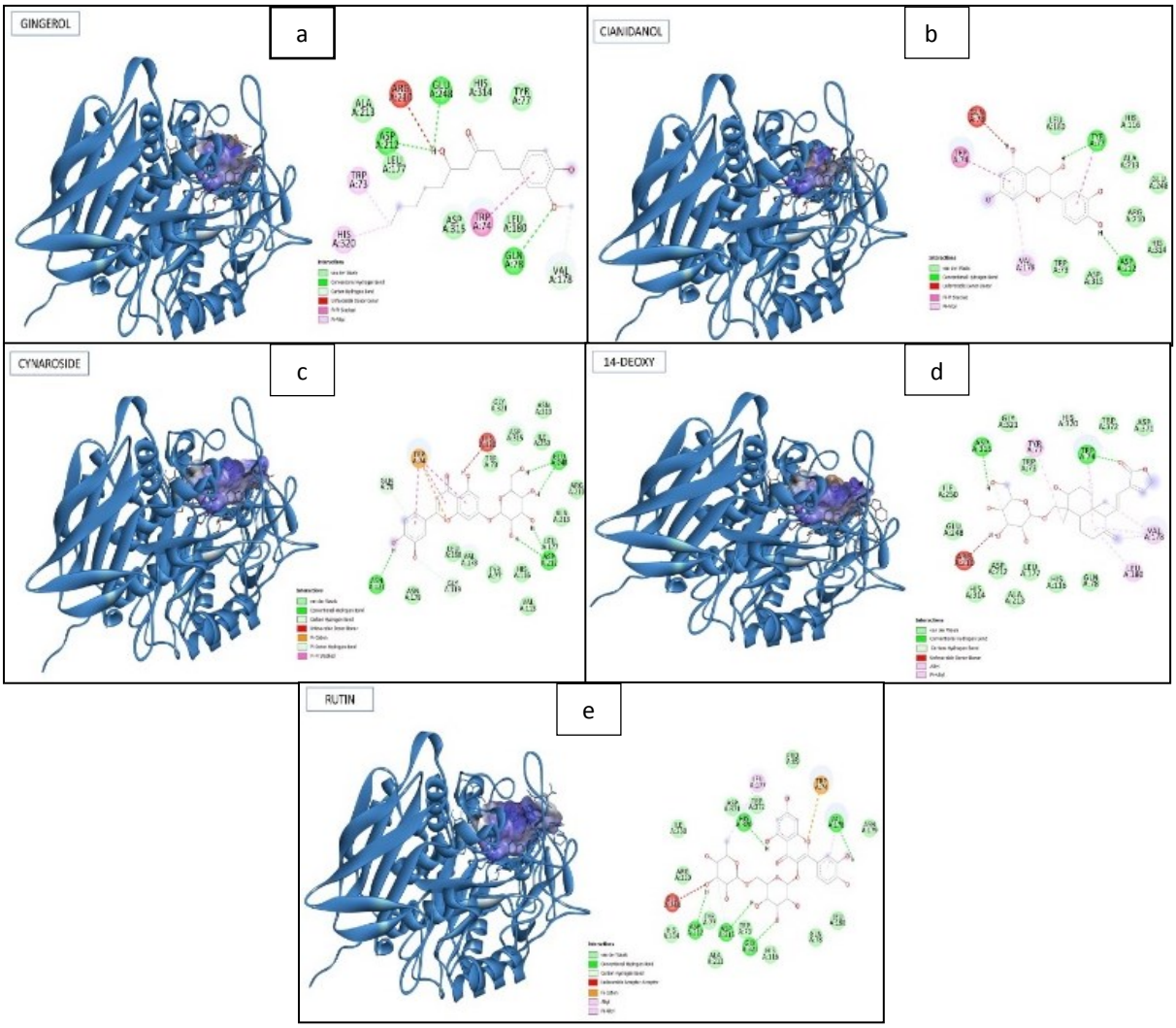

Figure 8. Visualisasi docking a. Gingerol, b. Cianidanol, c. Cynaroside, d. 14-deoxy-11,12-didehydroandrographiside, e. Rutin

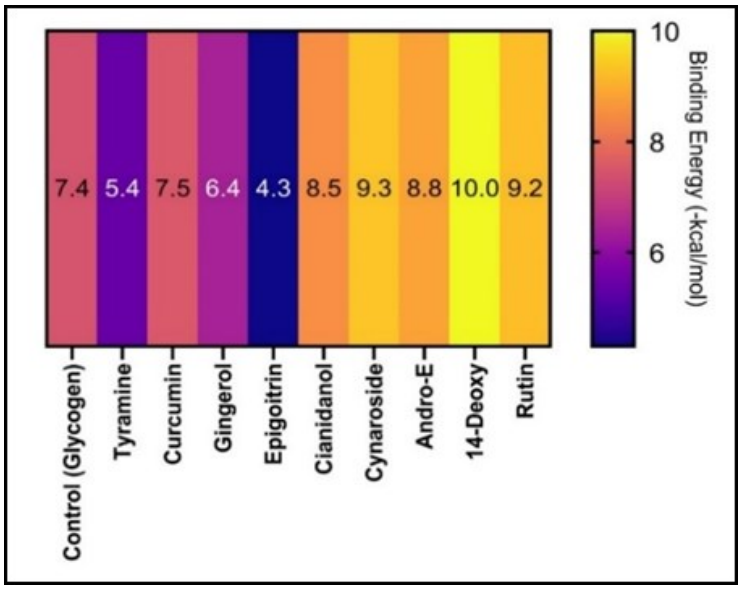

Figure 9. Binding Affinity

The complex of tyramine displays conventional hydrogen bonds at ASP(A):212, pi-pi stacked interactions at TYR(A):77, and van der Waals interactions at GLU(A):248, ALA (A):213, ARG(A):210, HIS(A):116, LEU (A):177, LEU(A):180, ASP(A):315, TRP(A):73, TRP(A):74, and GLN(A):78 (Figure 7a). The conventional hydrogen bond in curcumin is located at the amino acid ARG(A):210, the carbon-hydrogen bond is located at GLY (A):321, the pi-anion interaction is located at ASP(A):212, the pi-pi stacked interaction is located at TYR(A):77, and the carbon van der Waals interaction is located at TRP(A):73, TRP (A):74, HIS(A):314, HIS(A):116, HIS(A):216, LEU(A):180, LEU(A):177, GLU(A):248, ALA (A):213, ILE(A):250, TYR(A):166, and VAL (A):178 (Figure 7b). Epigotrin with a conventional hydrogen bond located at GLN (A):16 and van der Waals interactions located at ASP(A):227, while the carbon-hydrogen bond is located at LYS(A):242, LYS(A):223, and GLY (A):15, and the alkyl bond is located at ILE (A):223, LEU(A):226, LEU(A):229, and PRO (A):243 (Figure 7c). The docking of andrographidine E reveals conventional hydrogen bonds at ASN(A):179, GLN(A):78, and HIS(A):320, pi-pi stacked interactions at TRP(A):74 and TYR(A):77, attractive charge interactions at ASP(A):315, carbon-hydrogen bonds at VAL(A):17 and GLY(A):119, and van der Waals interactions at ALA(A):122, ASN (A):121, GLY(A):120, LEU(A):180, LEU (A):177, TRP(A):372, TRP(A):73, ARG(A):210, and ASP(A):212 (Figure 7d).

The results of the docking analysis demonstrated the binding interactions of different compounds with specific amino acid residues. For instance, gingerol compound (Figure 8a) was found to exhibit conventional hydrogen bonding with ASP(A):212, GLU (A):248, and GLN(A):78 amino acid residues, whereas unfavourable donor-donor bonding was observed with ARG(A):210 residues. In addition, pi-pi stacked bonding was detected in TRP(A):74, while pi-alkyl bonding was observed in TRP(A):73 and HIS(A):320 residues. Furthermore, van der Waals bonding was identified in ALA(A):213, LEU(A):177, HIS (A):314, TYR(A):77, ASP(A):315, LEU(A):180, and VAL(A):178 residues. Cianidanol with a conventional hydrogen bond located at TYR (A):77 and ASP(A): 212. Van der Waals bonding was detected in LEU(A):180, HIS (A):116, ALA(A):213, GLU(A):248, ARG (A):210, HIS(A):314, ASP(A):315, and TRP (A):73. Pi-pi stacked bonding was observed in TRP(A):74 and pi-alkyl in VAL(A):178 (Figure 8b). Similarly, conventional hydrogen bonding was observed in the cynaroside compound (Figure 8c) with ASN(A):121, ASP(A):212, and GLU(A):248 amino acid residues. Pi-Cation bonding was detected in TRP(A):74, while unfavourable donor-donor bonding was observed in HIS(A):320 residues. Moreover, pi-donor hydrogen bonding was observed in GLN(A):78 and GLY(A):119 residues, whereas van der Waals bonding was identified in ASN(A):179, LEU(A):180, VAL(A):178, TYR(A):77, HIS (A):116, VAL(A):113, LEU(A):177, ALA (A):213, ARG(A):210, GLY(A):321, TRP (A):73, ASP(A):315, ASN(A):313, and ILE (A):250 residues. In the case of the 14-deoxy-11,12-didehydroandrographiside compound (Figure 8d), conventional hydrogen bonding was detected in ASP(A):315 and TRP(A):74 amino acid residues, whereas unfavourable donor-donor bonding was identified in ARG(A):210 residues. carbon hydrogen bonding was detected in HIS (A):320, while alkyl bonding was observed in TYR(A):77, LEU(A):180, and VAL(A):178 residues. Additionally, van der Waals bonding was identified in GLY(A):321, ILE(A):250, GLU(A):248, HIS(A):314, ASP(A):212, LEU (A):177, ALA(A):213, HIS(A):116, GLN(A):78, TRP(A):372, and ASP(A):371 residues. Finally, for the rutin complex (Figure 8e), conventional hydrogen bonding was detected in HIS(A):320, ASP(A):212, ASP(A):315, GLY(A):321, and VAL(A):178 amino acid residues, whereas unfavourable donor-donor bonding was identified in GLU(A):248 residues. Moreover, pi -cation bonding was detected in TRP(a):74, while van der Waals bonding was identified in

ILE(A):250, ARG(A):210, HIS(A):314, TYR (A):77, ALA(A):213, TRP(A):73, HIS(A):116, GLN(A):78, LEU(A):180, ASN(A):179, ASP (A):371, and TRP(A):372 residues.

Binding Affinity

The protein selected from the modelling results was subjected to a docking process with a control compound, glycogen, and 9 other phytobiotic compounds. The interaction between glycogen and alpha-amylase resulted in a binding energy of -7.4 kcal/mol.

The interaction results demonstrate the binding affinity of all active compounds with α-amylase (Figure 9). The strongest binding was found to be 14-deoxy-11,12-didehydroandrographiside (-10.0 kcal/mol), while the weakest binding was observed in the epigoitrin complex (4.3 kcal/mol). When compared with the docking results of other active compounds, glycogen still exhibits relatively weak binding energy with curcumin (7.5 kcal/mol), cianidanol (8.5 kcal/mol), andrographidine E (8.8 kcal/mol), cynaroside (9.3 kcal/mol), and rutin (9.2 kcal/mol), while 14 -deoxy-11,12-didehydroandrographiside (-10.0 kcal/mol) displays the strongest binding affinity. Conversely, other compounds such as epigoitrin, tyramine, and gingerol exhibit lower binding energies than the control (Figure 9). Although cianidanol, cynaroside, and rutin exhibit higher binding energies than the control and possess conventional hydrogen bonds, which are strong, their interaction with the control is weak due to the presence of unfavourable bonds. Unfavourable bonds lead to weak binding and instability in proteins. An increase in unfavourable bonds in docking results indicates that the compound is ineffective in maintaining protein stability. Generally, unfavourable bonds result in molecular instability during dynamic molecular tests (Wibowo *et al*., 2021).

Similarly, while curcumin exhibits a higher binding energy than the control and possesses conventional hydrogen bonds, its interaction with andrographidine E is still stronger. Andrographidine E exhibits a binding affinity of 8.8 kcal/mol and possesses conventional hydrogen bonds, which makes it easier for the protein and compound to form a stable bond, as the interacting amino acids do not possess any unfavourable bonds (Marzouk *et al*., 2022). Therefore, andrographidine E is more stable than other candidate compounds.

Alpha-amylase is a vital enzyme that catalyzes the hydrolysis of the glycosidic bonds present in glycogen, resulting in the formation of maltose, a water-soluble disaccharide. It is known that active compounds in phytobiotics derived from herbs contain anti-nutrients that affect productivity and alpha-amylase in poultry. Alpha-amylase is an enzyme derived from salivary glands that breaks down the grains' starch into monosaccharides by increasing starch digestibility. This makes it more efficient to increase egg production in poultry. Computational analysis reveals that the protein alpha-amylase utilized in this study exhibits exceptional protein quality, with 99.3% of its amino acid residues situated in the preferred region and high stability levels. The protein interaction analysis of alpha-amylase from poultry directly connects to the glycogen breakdown pathway, which controls this *in silico* study. Molecular docking was conducted to examine the anti-nutrient effects of 9 active compounds in natural growth promoters used as additional feed for Mojosari ducks. The findings indicate that several 9 compounds have lower binding affinity than the enzyme's binding to glycogen.

None of the active compounds had stable binding types like the control. Results from clinical studies showed that *A. paniculate* given to rats provided weak inhibition of alpha-amylase (Subramanina *et al*., 2008). The weak hydrophobic binding types of the active compounds do not affect the performance of the alpha-amylase enzyme, as supported by *in vivo* data indicating increased egg production, an improvement in external and internal qualities, included parameters such as egg weight, shell weight, white egg volume, yolk volume, and yolk colour index in Mojosari ducks. The

primary possibility proposes that these active compounds help maintain insulin levels in duck blood by acting as agonists to glucagon-like peprtide1-receptor (GLP1R) or having anti-inflammatory properties on nitric oxide synthase (NOS). Andrographolide analogs such as andrographidine E can act as GLP1R agonists, which can enhance insulin secretion through protective pathways in beta cells, thereby affecting the reproductive performance and egg quality of Mojosari ducks (Damayanti *et al*., 2023). Furthermore, cynaroside, 14-deoxy-11,12 -didehydroandrographolide, and curcumin can be used as anti-inflammatory agents through the inhibition of the NOS protein pathway, which has been demonstrated by the stable blood profiles of Mojosari ducks, especially in their leukocyte and differential counts (Harahap *et al*., 2023).

CONCLUSION

The protein interaction analysis in poultry alpha-amylase has revealed its direct association with the glycogen breakdown pathway, which is the focus of this *in silico* study. In order to investigate the anti-nutrient effects of 9 active compounds found in natural growth promoters used as additional feed for Mojosari ducks, a molecular docking experiment was conducted. The results show that several compounds have weaker binding affinity than the enzyme's binding to glycogen. However, the hydrophobic binding types of these compounds do not seem to impact the performance of the alpha-amylase enzyme, as demonstrated by *in vivo* data. The most likely possibility is that these active compounds help regulate insulin levels in duck blood by acting as GLP1R agonists or having anti-inflammatory effects on nitric oxide synthase (NOS).

CONFLICT OF INTEREST

The author declares no conflict of interest.

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