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Research Article

Potency of moringa (*Moringa oleifera* L.) leaves extract containing quercetin as a depigmentation agent inhibiting the tyrosinase enzyme using *in-silico* and *in-vitro* assay

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Abstract

Hyperpigmentation is a disorder of facial skin pigments due to an increase in the process of melanogenesis, which can cause a darkening of skin color. A flavonoid compound with potential as a skin-lightening agent is quercetin, commonly found in *Moringa oleifera* L. leaves. This study aims to determine quercetin's affinity and molecular mechanism on tyrosinase enzyme target proteins using an *in-silico* molecular docking method. Docking of quercetin with the tyrosinase enzyme produced a bond energy value of -7.08 kcal/mol. In comparison, the tropolone as a native ligand with the tyrosinase enzyme produced -4.79 kcal/mol. Quercetin has a strong affinity for the tyrosinase enzyme, indicated by the bond energy results from docking. Quercetin extraction from *Moringa oleifera* L. leaves using three different extraction methods: maceration, soxhlation, and reflux were made. The chromatogram from the TLC-Densitometry method showed the identification result in maceration and soxhlation extract containing quercetin, while reflux extract did not contain quercetin. The highest quercetin was obtained in the maceration method with a level of 21.57% w/w, while the soxhlation received quercetin as much as 18.49% w/w. *In-vitro* tests were carried out using a spectrophotometric method using a comparison of kojic acid. The *in-vitro* test found that IC_{50} from kojic acid was 48.90 µg/mL and IC_{50} of the extract from moringa leaf maceration of 115.36 µg/mL. Based on this research, quercetin compounds in *Moringa oleifera* L. leaves from maceration can potentially be skin-lightening agents.

Keywords

In-Vitro, *Moringa oleifera* L. leaves, Molecular Docking, Quercetin, Tyrosinase

Introduction

Most tropical country women assume that light-colored skin, free of brownish stains, is the hallmark of beautiful skin. Therefore efforts to get lighter skin have become a trend (Baran and Howard 1998). Frequent and prolonged exposure to UV light causes hyperpigmentation problems in the skin. Hyperpigmentation is a common skin pigment disorder that occurs due to enhanced melanogenesis which cause skin color darkening (Bino et al. 2018).

The process of melanogenesis or biosynthesis of melanin requires tyrosinase. Tyrosinase catalyzes two reactions: hydroxylation of L-tyrosine to Dihydroxyphenylalanine (DOPA) and oxidizes DOPA to DOPAquinone (Sugumaran

and Barek 2016). The conversion of L-tyrosine to L-DOPA by tyrosinase is a rate-limiting stage in the process of melanin synthesis (Videira et al. 2013). One way to prevent skin hyperpigmentation is by inhibiting the formation of melanin by inhibiting tyrosinase enzyme activity (Nerya et al. 2004).

The precaution for hyperpigmentation is to use skin-lightening cosmetic products. Substances commonly used for lightening include arbutin, hydroquinone, ascorbic acid, kojic acid, and kojic acid derivative compounds. However, some of these compounds have dangerous side effects related to carcinogenesis and mutagenesis (Couteau et al. 2016). Thus, efforts are needed to develop skin lightening from natural materials to be safer in its use.

Based on empirical data, *Moringa oleifera* L. (Moringaceae) leaves are usually used for skincare as skin nutrition, anti-aging, moisturizing, and sunscreen (Meireles et al. 2020). The most abundant flavonoid compound in *Moringa oleifera* L. leaves is quercetin (Vergara-Jimenezet al 2017). Quercetin is thought to have an anti-tyrosinase activity to brighten the skin (Choi and Shin 2016). Based on this, a preliminary test is needed to determine quercetin activity as a skin whitening agent using molecular docking and *in-vitro* testing.

Experimental

Material and instrument

A computer with windows 10, 64 bit specifications that is equipped with Hyperchem 8, Chimera 1.10.1, and AutoDock Tools consisting of the Autodock 4.2 and Autogrid programs. Camag TLC Automatic Sampler (ATS 4), TLC-scanner 3 Camag, UV-spectrophotometry (Shimadzu), GF 254 silica gel plates (Merck), toluene for analysis emsure (Merck), ethyl acetate (Merck), formic acid (Merck), methanol (Merck), solution of phosphate buffer, kojic acid (Sigma), tyrosinase enzyme (Sigma) and L-DOPA (Sigma). The material used in the docking study is the tyrosinase enzyme (PDB ID: 2Y9X), which was downloaded from http://www.rcsb.org/pdb/home/home.do. Then, the 3-dimensional structure of quercetin and kojic acid was downloaded from https://pubchem.ncbi.nlm.nih.gov/compound.

Plant material

Moringa leaves were obtained from Badung District, Bali Province of Indonesia. The plant was identified by the Indonesian Institute of Sciences Bali Botanic Garden with the certificate number of B-5718/III/KS.01.03/7/2021.

In-silico test

3-Dimensional protein preparation targeting tyrosinase enzyme was carried out using the Chimera 1.10.1 program by separating the 3-dimensional structure of the tyrosinase protein from the native ligand. The molecular docking method was validated by docking the native ligand to the tyrosinase enzyme target protein using the AutoDock Tools application (Autodock 4.2 and Autogrid). Optimization of the 3-Dimensional Structure of Quercetin Compounds with the Hyperchem program 8. The quercetin that has been optimized is docking to the target protein of the tyrosinase enzyme prepared using the Autodock Tools application with the docking process according to the method validation.

Analysis – *in-silico* testing

Molecular docking results were bond energy. The bond energy value indicates the bond strength between the compound and the target protein. The lower the bond energy value, the stronger and more stable the bond.

Extraction of *Moringa oleifera* L.

Moringa oleifera L. leaves were collected, sorted, and thereafter then dried at room temperature and eventually mashed and sifted using a 60 mesh sieve. Moringa leaf powder was extracted by maceration, soxhlation, and reflux methods. Moringa leaf powder was weighed at 25 grams for each extraction method. Then each extraction process was carried out using a solvent consisting of 500 mL methanol and 100 mL 1.2 N HCl. The extract obtained was evaporated by the solvent with a rotary evaporator and oven (Pakade et al. 2013; Manasa et al. 2014).

Identification of quercetin in extract

Identification of quercetin compounds and determination of quercetin levels in extracts were carried out by TLC-densitometry method using standard quercetin compounds with the mobile phase of toluene: ethyl acetate: formic acid (5: 4: 0.2). The plate was scanned at a wavelength of 265 nm using a densitometer instrument (Sudjarwo et al. 2019).

In-vitro test

The *in-vitro* test was done by measuring the dopachrome uptake first to obtain the maximum dopachrome wavelength. This measurement was carried out by measuring the absorbance of the L-Dopa solution, which was added with the enzyme tyrosinase in phosphate buffer solution, which was incubated for 15 minutes (Lukitaningsih and Holzgrabe 2014). Testing the inhibitory potency of quercetin on the tyrosinase enzyme was carried out by comparing its inhibition with kojic acid. The non-inhibiting test solution is made by adding phosphate buffer and L-DOPA solution into a test tube and incubating at room temperature for 15 minutes. Tyrosinase solution was added and homogenized using vortex, then incubated for 15 minutes at room temperature again. A UV-Vis spectrophotometer measured uptake at λ max. The test solution with inhibitors was made to add buffer phosphate, L-DOPA, and inhibitors to the test tube and incubated at room temperature 15 minutes. Tyrosinase solution was

added and homogenized by using vortex, then incubated again for 15 minutes at room temperature (Laksmiani and Nugraha 2019). Uptake was measured at the measurement wavelength. Calculated % inhibition of the two compounds tested. The measurement of IC_{50} was done by varying the concentration of Moringa leaf extract used, from 50–150 µg/mL (ppm) and kojic acid, from 20–100 µg/mL (ppm). Uptake was measured and calculated as % inhibition. The plot in the curve between Moringa leaves extracts concentration and kojic acid vs % inhibition and IC_{50} could be calculated.

Analysis of *in-vitro* test

The absorbance value is used to calculate % inhibition. Percentage of inhibition data were used to determine IC_{50} values by plotting moringa leaves extracts concentrations, kojic acid concentration vs % inhibition. The linear equation obtained from the curve is used to calculate the IC_{50} value of quercetin from moringa leaves extracts and kojic acid which have tyrosinase inhibitory activity of 50%.

Result and discussion

Quercetin Inhibit Tyrosinase Using Molecular Docking

Evaluation of *in-silico* skin-lightening activity of quercetin using the molecular docking method was carried out through several stages, starting from preparing a 3-dimensional structure database for the quercetin test compound downloaded at https://pubchem.ncbi.nlm.nih.gov/ and optimized with the Hyperchem 8 program. The target protein was prepared using the Chimera 1.10.1 program. Validation of the molecular docking method and then docking quercetin to the target protein was carried out using the AutoDock Tools application, which consisted of Autodock 4.2 and Autogrid programs.

Target protein preparation aimed to obtain the target protein structure without native ligand so that pockets are available for docking processes and obtain native ligand structures (Ismaya et al. 2019). The process of protein preparation was also carried out by removing water molecules (H_2O), which aim to leave amino acids in the target protein so that when the docking process interacts, only test compounds with amino acids (Huey et al. 2012).

The validation of the molecular docking method was done by redocking the tropolone as a native ligand to the target protein tyrosinase enzyme that had been prepared (Hawkins et al. 2008). The method gets valid data if the value of RMSD ≤ 3 Å is obtained (Jain and Nicholls 2008). The process of redocking the target protein of the tyrosinase enzyme with its native ligand results in an RMSD value of 2.05 Å. The result of the visualization is shown in Figure 1. The RMSD value was below 3 Å, which indicated that the molecular docking method was valid.

Optimization of quercetin compounds carried out by the semi-empirical computational method AM1 (Austin Model

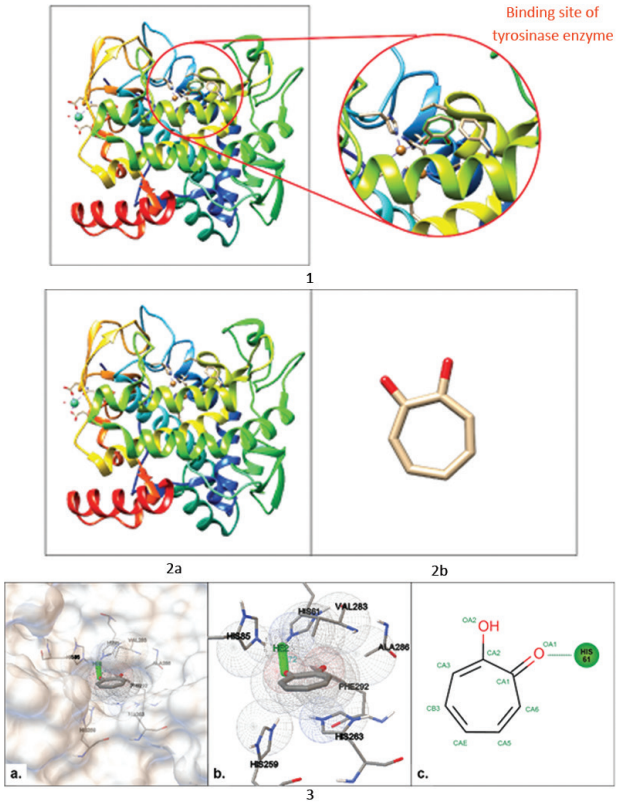

Figure 1. Redocking visualization between tyrosinase enzyme and native ligand. **1**: The structure of the tyrosinase enzyme (PDB ID: 2Y9X), **2a**: tyrosinase enzyme structure without native ligand, **2b**: native ligand (tropolone) structure, **3a**: redocking conformation, **3b**: 3D interaction of amino acid residues from tyrosinase enzyme with native ligand, **3c**: 2D interaction of amino acid residues from tyrosinase enzyme with native ligand.

1) includes single-point calculation processes and geometry optimization (Laksmiani et al. 2018). The energy obtained during single-point calculations and geometry optimization on quercetin structures were -3699.53 kcal/mol and -3711.73 kcal/mol, respectively. The process of compound structure optimization was successful if the geometry optimization energy had a lower value than the single-point calculation. Based on this, the optimization process had been successful, and a 3-dimensional quercetin structure had been optimized. A low energy value indicated an interaction in the form of a greater attraction between atoms. In contrast, the repulsion between atoms became less so that the conformation of the compound obtained was more stable than before the optimization process was conducted (Syahputra et al. 2021)

Docking between quercetin and tyrosinase target protein produces a bond energy value of -7.08 kcal/mol (Table 1). At the same time, the tropolone (native ligand) interact with the target protein tyrosinase enzyme produces a bond energy value of -4.79 kcal/mol (Table 1). Table 1 demonstrate binding energy value of active compound in *Moringa oleifera* L. leaves, quercetin and kojic acid as compound that been proven as depigmentation agent bond to tyrosinase enzyme as target protein. The lower the bond energy value, the better the stability or bond strength of the

Table 1. The quercetin and kojic acid bond energy with tyrosinase protein.

Target Protein	Ligand	Energy Bond (kkal/mol)	Hydrogen bond	Cluster (Ligand-Protein)
Tyrosinase (2Y9X)	Kojic acid	-5.03	HIS259 HIS296	O-HE2 O-HE2
	Quercetin	-7.08	HIS61 HIS85	O-HE2 O-HE2
	Native Ligand	-4.79	HIS61	OA1-HE2

test compound with the target protein, so that the affinity for binding is higher so that the affinity of quercetin against the tyrosinase enzyme target protein is higher than the affinity of the native ligand with tyrosinase enzyme as a target protein (Laksmiani et al. 2018; Fakhrudin et al.2020).

The types of bonds that can be calculated in the AutoDock Tools program are hydrogen bonds, ionic bonds, hydrophobic interactions, aromatic interactions, and Van der Walls interactions, but only hydrogen bonds could be visualized in the program. Although other interaction and bonding models could not be visualized, they still affected the bond energy between the compound and the target protein. Hydrogen bonding was the most likely bond because quercetin was an organic compound that had –OH and –H groups in its structure (Fig. 2). The bond strength of hydrogen bonds was 1–10 kcal/mol and reversible (Kitchen et al. 2004).

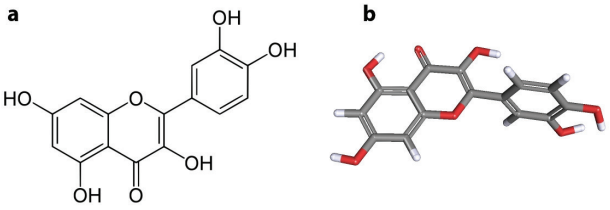

Figure 2. 2D Chemical structure of quercetin (**a**), 3D chemical structure of quercetin (**b**).

The hydrogen bonds formed a strong interaction between quercetin, kojic acid, and native ligands with tyrosinase. The interaction model of the test compound with the target protein was shown in Fig. 3, and Fig. 4. The amino acid residues involved in forming hydrogen bonds between ligands and tyrosinase were His259 and His296 (kojic acid), His61 and His85 (quercetin), and native ligands form bonds with tyrosinase at His61. The native ligand and quercetin had the same hydrogen bonds at amino acid residue His61 in tyrosinase. Quercetin forms bonds with higher affinity than native ligands when viewed from a lower (negative) bond energy than the binding energy formed by protein-native ligand complexes. The molecular docking results

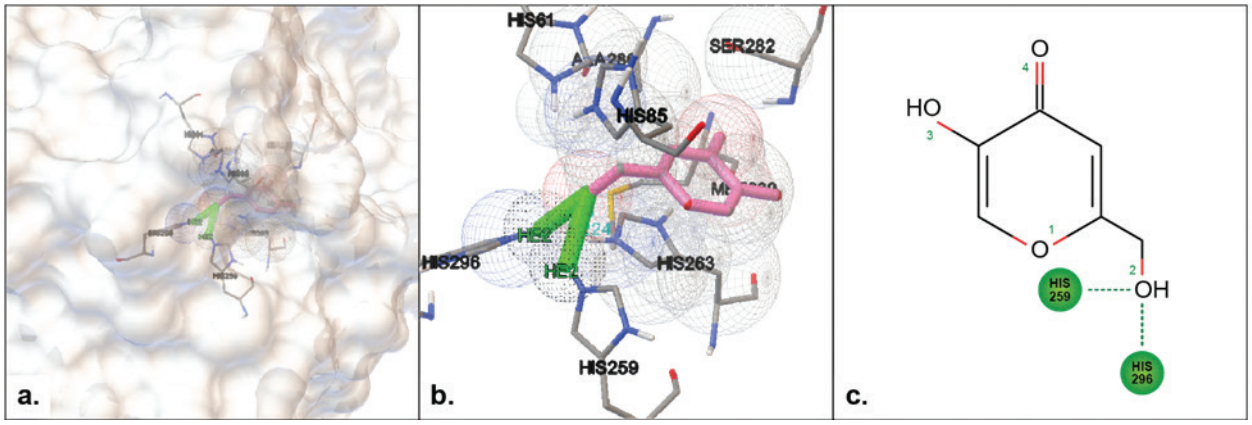

Figure 3. The interaction of hydrogen bonds between the target protein of the tyrosinase with kojic acid. **a**: docking conformation kojic acid to tyrosinase, **b**: 3D interaction visualization of tyrosinase amino acid residues with kojic acid, **c**: 2D interaction visualization of tyrosinase amino acid residues with kojic acid.

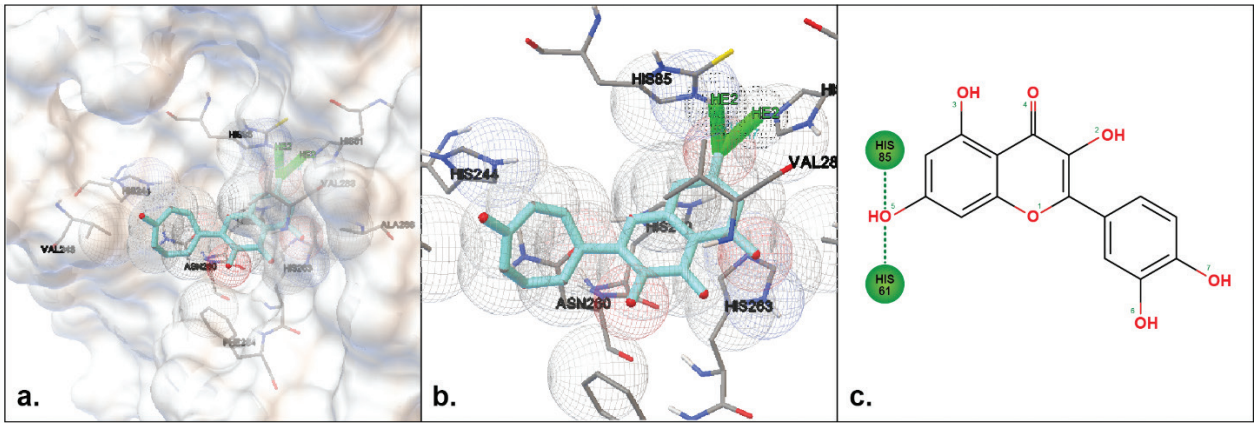

Figure 4. The interaction of hydrogen bonds between the target protein of the tyrosinase with quercetin. **a**: docking conformation between quercetin and tyrosinase, **b**: 3D interaction visualization of tyrosinase amino acid residues with quercetin, **c**: 2D interaction visualization of tyrosinase amino acid residues with quercetin.

showed that quercetin slows down the melanogenesis through tyrosinase inhibition.

Identification of quercetin in the extract

Identification of quercetin from the three extracts was carried out using the TLC densitometry method using the mobile phase in the form of toluene: ethyl acetate: formic acid (5: 4: 0.2). Scanned plate at the maximum quercetin wavelength of 265 nm. The presence of quercetin in each

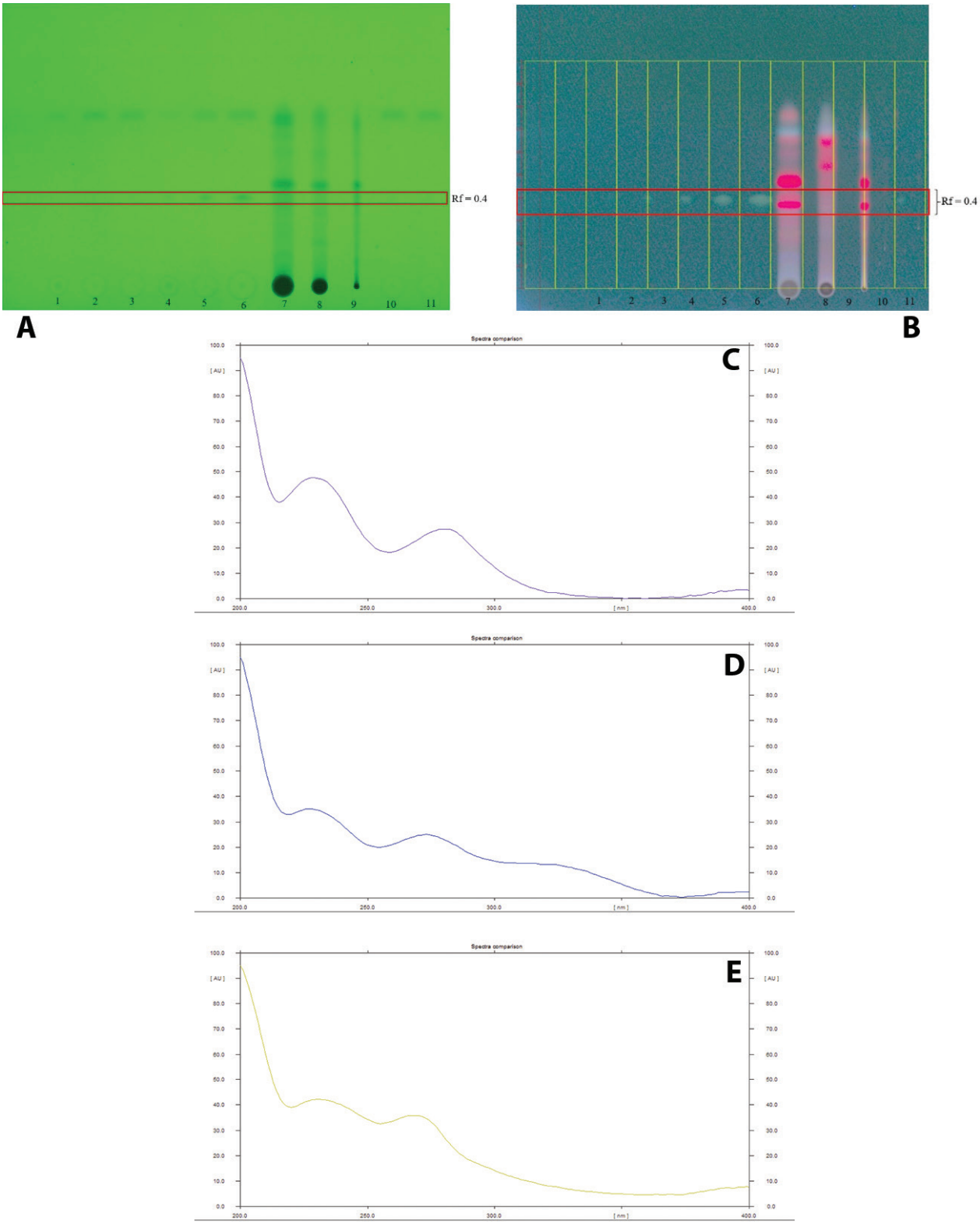

Figure 5. TLC-chromatogram of Moringa Leaves extract at UV 254 nm (**A**) and UV 366 nm (**B**). Quercetin standard 100, 200, 400, 800, 1600 and 3200 ng (1–6); Moringa Leaves Maceration extract (7); reflux extract (8), soxhlation extract (9); quercetin standard 800 ng (10–11); UV spectrum of spot with Rf 0.4 from quercetin standard (**C**); UV spectrum of spot with Rf 0.4 from maceration extract of Moringa Leaves (**D**); UV spectrum of spot with Rf 0.4 from soxhlation extract of Moringa Leaves (**E**).

extract of Moringa leaves with different extraction processes can be seen in Figure 5. The identification results obtained in maceration extract contained the most quercetin at 21.57% w/w, soxhlation method obtained quercetin as much as 18.49% w/w, while the reflux extraction method did not contain quercetin. The UV spectrum similarity between quercetin standard and quercetin from maceration and soxhlation extract strengthens the presence of quercetin in the extract.

Fig. 6 showed the AUC (Area Under the Curve) data of quercetin standard. AUC data was obtained by measuring

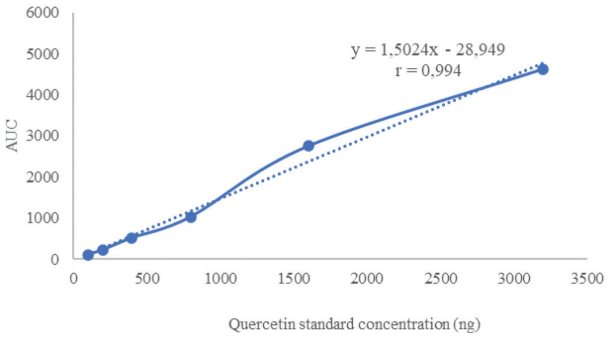

Figure 6. The regression curve of the relationship between AUC and series concentration of quercetin standard.

the absorbance of each Quercetin standard solution using TLC densitometry with mobile phase toluene: ethyl acetate: formic acid (5: 4: 0.2).

Table 2 showed the quercetin content in moringa leaves extracts. AUC data were obtained through absorbance measurements using TLC Densitometry with mobile phase toluene: ethyl acetate: formic acid (5: 4: 0.2). Based on

Table 2. Quercetin contained in moringa leaves extracts AUC.

Solution	AUC
Maceration Extract	1591.3
Reflux Extract	–
Soxhlation Extract	1360.2

the regression calculation, the quercetin level in the extract obtained from maceration was 24.45% w/w and in the soxhlation Moringa leaves extracts was 20.95% w/w.

Quercetin in Moringa Leaves Extract Repress Tyrosinase by *In-vitro* Assay

The maximum wavelength of dopachrome (Fig. 7) was done by measuring the absorbance of the dopachrome solution. The dopachrome solution was made by mixing L-DOPA solution, phosphate buffer, and tyrosinase enzyme, which was incubated for 15 minutes. The incubation solution then measured its absorbance in a wavelength range of 450–550 nm. The maximum wavelength of dopachrome obtained is 480 nm.

Fig. 8 and Fig. 9 demonstrated the percentage of tyrosinase enzyme inhibition of kojic acid and *Moringa oleifera* L. leaves extract. This % inhibition data was obtained through

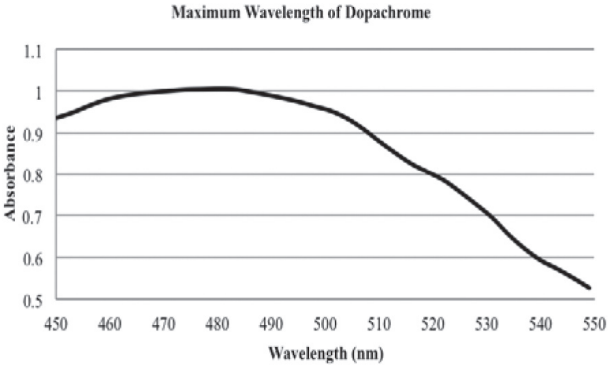

Figure 7. Dopachrome spectrum using spectrophotometry UV-VIS.

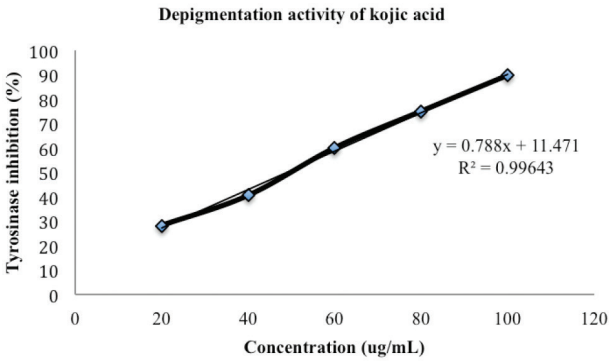

Figure 8. Tyrosinase inhibition (%) curve of kojic acid.

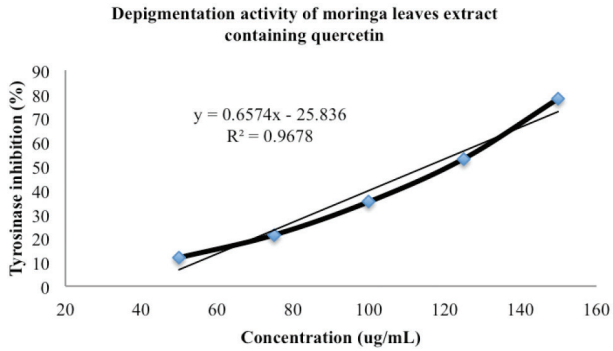

Figure 9. Tyrosinase inhibition (%) curve of moringa leaves extract.

calculations [(absorbance without inhibitors – absorbance with inhibitors)/absorbances without inhibitor] × 100%. Absorbance without inhibitors was the maximum absorbance of dopachrome. In contrast, absorbance with inhibitors was the absorbance of L-DOPA solution plus phosphate buffer, tyrosinase enzyme, and inhibitors (kojic acid or testing extract solution) incubated for 15 minutes. The incubation solution was measured for its absorbance and calculated to get % inhibition. The data was then used to curve of the relationship between % tyrosinase inhibition and inhibitor concentration to get a regression equation. Through this regression equation IC_{50} was calculated from kojic acid.

The *in-vitro* test was carried out by measuring the suppression of the work of the tyrosinase enzyme. This test

was based on measuring the substance's color from orange to red, dopachrome, which results from L-DOPA oxidation by tyrosinase (Lukitaningsih and Holzgrabe 2014). The presence of inhibitors caused a decrease in production from dopachrome, characterized by reducing the intensity of the orange color that occurs (Thanigaimalai et al. 2017). The inhibitory activity of the tyrosinase enzyme was characterized by IC_{50} value, the concentration of inhibitors, which inhibit 50% of the tyrosinase (Laksmiani and Nugraha 2019). *In-vitro* evaluation began with measuring the maximum wavelength of dopachrome, and this wavelength will be used as the measurement wavelength. The results of dopachrome maximum wavelength measurements could be seen in Fig. 7. Based on the analytical results, the maximum dopachrome wavelength of 480 nm with an absorbance of 1.007. IC_{50} were carried out by making a series of concentrations of kojic acid and moringa leaf extracts. The two solutions were then used to calculate the % inhibition. A linear regression equation was made, which could be used to determine IC_{50} from kojic acid and maceration extracts of moringa leaf (Laksmiani and Nugraha 2019). Based on these data, the IC_{50} of kojic acid was 48.90 µg/mL, while IC_{50} from the Moringa leaves extracts was 115.36 µg/mL.

Inhibition of the activity of this melanogenesis enzyme had the effect of increasing skin brightness (Ebanks et al. 2009). Based on the results, quercetin has potent activity as a skin-lightening agent. Quercetin in Moringa leaves extract disrupts the melanogenesis mechanism through the tyrosinase pathway inhibiting the formation of eumelanin and providing a skin brightening effect.

Conclusion

Quercetin had a strong affinity for the tyrosinase target protein, as indicated by the bond energy value of the docking product. Quercetin had a greater affinity than native ligands. *Moringa oleifera* L. leaves extract containing quercetin had activity as skin lightening *in-silico* and *in-vitro* assay through tyrosinase inhibition.

Conflict of interest

The authors declare no conflict of interest.

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