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The activity of avocado (*Persea americana* Mill.) seed extract containing catechin as a skin lightening agent

[Actividad del extracto de semilla de aguacate (*Persea americana* Mill.) conteniendo catequina como agente aclarante de la piel]

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Abstract

Context: Indonesia is a tropical country with high UV rays. UV rays can increase melanin synthesis in the skin and cause the skin to become darker and hyperpigmented. One way to overcome this problem is the use of skin lightening agents through the mechanism of tyrosinase inhibition. Flavonoids have antioxidant activity and inhibit the process of melanogenesis. Also, avocado seeds contain secondary metabolites of flavonoids in the form of catechin.

Aims: To determine the potential and activity of catechin as skin lightening agent against the target protein, tyrosinase, by *in silico* test using molecular docking method and *in vitro* test compared to kojic acid.

Methods: *In silico* assay was carry out using a computational method with autodock 4.2 program to demonstrate the affinity of active compound (catechin) with tyrosinase as the target protein by evaluating the binding energy value. Inhibition of tyrosinase is one way to inhibit the formation of melanin, so the skin becomes brighter. Spectrophotometry method was conducted to measure the absorbance of dopachrome and calculate the percentage of tyrosinase inhibition to be altered as IC_{50}.

Results: The energy values of catechin and kojic acid in tyrosinase enzymes were -7.64 kcal/mol and -5.03 kcal/mol, respectively. The energy value of the catechin bond was smaller than kojic acid in the tyrosinase. The bond energy value showed that catechin had greater potential than kojic acid as a skin lightening agent by inhibiting tyrosinase *in silico* using the molecular docking method. The IC_{50} value from ethyl acetate extract of avocado seeds disrupt the tyrosinase using the *in vitro* test was 93.02 ± 1.98 µg/mL, while IC_{50} kojic acid was 48.67 ± 0.1 µg/mL.

Conclusions: Avocado seeds extract containing catechin has a potential activity as a lightening agent by inhibiting the tyrosinase. Further research must be done to fractionation the extract to get a significant effect.

Keywords: avocado seed; *in silico*; *in vitro*; tyrosinase inhibitor.

Resumen

Contexto: Indonesia es un país tropical con altos rayos UV. Los rayos UV pueden aumentar la síntesis de melanina en la piel y hacer que la piel se vuelva más oscura e hiperpigmentada. Una forma de superar este problema es el uso de agentes para aclarar la piel a través del mecanismo de inhibición de la tirosinasa. Los flavonoides tienen actividad antioxidante e inhiben el proceso de melanogénesis. Además, las semillas de aguacate contienen metabolitos secundarios de flavonoides en forma de catequina.

Objetivos: Determinar el potencial y la actividad de la catequina como agente para aclarar la piel contra la proteína objetivo, tirosinasa, mediante prueba *in silico* utilizando el método de acoplamiento molecular y la prueba *in vitro* en comparación con el ácido kójico.

Métodos: El ensayo *in silico* se llevó a cabo utilizando un método computacional con el programa Autodock 4.2 para demostrar la afinidad del compuesto activo (catequina) con la tirosinasa como la proteína diana mediante la evaluación del valor de energía de unión. La inhibición de la tirosinasa es una forma de inhibir la formación de melanina, por lo que la piel se vuelve más brillante. El método de espectrofotometría se realizó para medir la absorbancia del dopacromo y calcular el porcentaje de inhibición de la tirosinasa que se alterará como IC_{50}.

Resultados: Los valores de energía de catequina y ácido kójico en las enzimas tirosinasa fueron -7,64 kcal/mol y -5,03 kcal/mol, respectivamente. El valor energético del enlace de catequina fue menor que el ácido kójico en la tirosinasa. El valor de la energía de enlace mostró que la catequina tuvo un mayor potencial que el ácido kójico como agente para aclarar la piel al inhibir la tirosinasa *in silico* utilizando el método de acoplamiento molecular. El valor IC_{50} del extracto de acetato de etilo de las semillas de aguacate alteró la tirosinasa usando la prueba *in vitro* que fue 93,02 ± 1,98 µg/mL, mientras que para el ácido kójico fue 48,67 ± 0,1 µg/mL.

Conclusiones: El extracto de semillas de aguacate que contiene catequina tiene una actividad potencial como agente aclarante al inhibir la tirosinasa. Se deben realizar más investigaciones para fraccionar el extracto para obtener un efecto significativo.

Palabras Clave: *in silico*; *in vitro*; semilla de aguacate; tirosinasa inhibidor.

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INTRODUCTION

Exposure to UV rays on the skin for a too long time causes interference with the skin (Lee et al., 2014). The skin naturally forms melanin, which functions to protect the skin from the adverse effects of UV radiation (Holick, 2016). However, excessive exposure to UV light results in increased melanin synthesis in the skin (Ya et al., 2015; Pillaiyar et al., 2017). Increased melanin synthesis in the skin causes the skin to become dark in color (Nieuwpoort et al., 2004; Brenner and Hearing, 2008). Cosmetics for skin whitening agent purpose, contain compounds that act as tyrosinase inhibitors. Tyrosinase is an enzyme that acts as a catalyst in the hydroxylation reaction of monophenol into diphenol and oxidation of diphenol into quinone in the process of melanogenesis (Fenoll et al., 2001; Gillbro and Olsson, 2010). Inhibition of these enzymes reduce the effect of skin darkening, so it increases the brightness of the skin (Laksmiani and Nugraha, 2019).

The active agent used as skin lightening is hydroquinone, kojic acid, mercury and arbutin. These compounds can cause side effects on the skin, such as allergies, irritation, dermatitis, burning sensation on the skin, and cancer in long term use (Celine and Coiffard, 2016; Gajjala et al., 2016; Kanan et al., 2017). Based on this, alternative active ingredients need to have the same mechanism of action and are safer for health. Research on natural ingredients as active compounds are developed rapidly, especially as a natural skin-lightening agent. The possibility of side effects is slighter than the use of synthetic ingredients. Flavonoids are natural polyphenols that are found in leaves, stems, and flowers. These metabolites have the ability as a skin lightening agent by directly inhibiting tyrosinase activity in the melanogenesis process. Flavonoid bonding with copper, hydroxyl position in ring A and ring B, which is very decisive, and antioxidant effects are reported to play a role in inhibiting the tyrosinase enzyme. Some natural compounds with flavonoids and act as tyrosinase inhibitors are *Licorice* root containing glabrene compounds with IC_{50} = 3.5 µg/mL, *Camellia sinensis* leaf extract with IC_{50} = 20.69 µg/mL, and wood of *Artocarpus heterophyllus* with IC_{50} = 50 µg/mL (Nerya et al., 2003; Fu et al., 2005; Chang, 2012). Further research is needed on other natural compounds containing flavonoids as active compounds from new and potential natural substances inhibiting the tyrosinase enzyme. It can be developed as a natural skin lightening agent.

Avocado seeds (*Persea americana* Mill.) contain flavonoids, namely catechins, which were compounds with high antioxidant activity (Alagbaoso et al., 2015). Flavonoids are compounds that generally have activity as a skin lightening agent. Catechin activity as a skin whitening agent can be known by a preliminary test using molecular docking *in silico* and confirm by *in vitro* test to certain the activity of avocado seed catechin as a skin whitening agent. The objectives of this research are to evaluate the affinity of catechins contained in avocado seeds extracts using molecular docking with the tyrosinase as the target protein and to describe tyrosinase inhibitor activity of avocado seeds extracts by *in vitro* assay and determine the IC_{50} of the extracts compared to kojic acid.

MATERIAL AND METHODS

Material and instrument

Three-dimensional structure of melanogenesis enzyme target protein, tyrosinase (2Y9X) for *in silico* was used, which was downloaded from http://www.rcsb.org/pdb/home/home.do. Then, the 3-dimensional structure of the catechins to be used was downloaded from https://pubchem.ncbi.nlm.nih.gov/compound/. Ethyl acetate, acetone, 95% ethanol, methanol, silica gel TLC plate GF 254 nm were purchased by Merck, catechin standard (Sigma). *In vitro* assays used avocado seeds extract containing catechin as a sample to be tested, L-DOPA solution (Sigma-Aldrich D9628), tyrosinase from mushroom (Sigma-Aldrich T3824) and kojic acid (Sigma-Aldrich K3125). The equipment used in the *silico* test was a computer set with Windows 10 64-bit specifications equipped with Autodock 4.2 program, Chimera 1.10.1, and Hyperchem 8. Identification of catechin compounds was performed using a degree of alarm, measuring flask, horn spoon, drop pipette, vial bottle, measuring cup, measuring pipette, bulb filler, chamber,

TLC-Densitometry instrument, and UV-Vis spectrophotometer (UV Mini-1240) Shimadzu.

Preparation of avocado seeds (*Persea americana* Mill.)

Samples of avocado seeds were collected from Rendang village, Karangasem regency (8°25'44.173"S, 115°25'45.227"E), Bali, Indonesia. This plant was identified by the Indonesian Institute of Science in Baturiti village, Tabanan regency, Bali, Indonesia, with register number B-485/IPH.7/AP/VI/2019. Avocado seeds were peeled and then washed thoroughly. Avocado seeds were thinly sliced to facilitate drying. The avocado seed slices were then dried in an oven with a temperature of 60°C. Sliced avocado seeds were dried and then mashed with a blender until it became powder with the particle size (80 mesh) of avocado seeds.

Samples extraction

Avocado seed powder was extracted with the maceration method using a variety of solvents. The solvents used were ethyl acetate, acetone, and 95% ethanol with a ratio of powder:solvent (1:10 w/v). The avocado seed powder each weighed 100 g and then put it in each jar. Then macerated with each solvent used as much as 1000 mL and stirred using a stirrer (Corning PC-420D, USA) slowly. The extract was left to stand for 24 h, then filtered to obtain a macerated. Remacerate with each solvent as much as 1000 mL and let stand for 24 h. The extract was filtered until extracts of re-maceration results were obtained. The maceration extract and re-maceration results were then mixed and evaporated by using a rotary evaporator (EYELA, Tokyo, Japan). The evaporation extract was then evaporated again in an oven with a temperature of 60°C until a concentrated extract was obtained.

Identification of catechins in extracts

Identification of catechin compounds in each of the concentrated extracts of avocado seeds with various solvents (ethyl acetate, acetone, and 95% ethanol) was carried out using the TLC-Densitometry method. Concentration standard solution of catechins was 1 mg/mL, concentration series solution with variations in concentrations of 20, 40, 80, 160, and 320 ppm. Preparation of stationary phase, namely TLC silica gel GF 254 plate with a size of 20 × 10 cm. The mobile phase used was toluene:ethyl acetate:formic acid:methanol (3:6:1.6:0.4, v/v/v/v) as much as 35 mL. Preparation of sample solution was carried out by weighing 1 g dried extract then dissolving it in 10 mL of methanol. The series solution, and the three sample solutions that have been prepared, were then bottled using an automatic sampler with a volume of 5 µL serial solution, and the volume of the bottled sample solutions were 5, 10, and 15 µL on the TLC plates, which were washed with methanol and activated at 110°C for 15 minutes. The TLC plates were eluted using the mobile phase of toluene:ethyl acetate:formic acid:methanol (3:6:1.6:0.4, v/v/v/v) as much as 35 mL. After the elution was finished, the TLC plate was scanned in TLC-densitometry with a wavelength of 200 nm to 400 nm.

***In silico* assay**

In silico test was carried out with several steps (Gurjar and Pal, 2020). Started, the optimization of the 3-dimensional structure of catechin as the test compounds. Optimization process using the HyperChem 8 program on a 3-dimensional structure of catechin compounds complete with hydrogen atoms with single point calculation stages and geometry optimization. Geometry optimization aims to get the best molecular conformation and stable configuration value with a lower total energy value compared to a single point. And then, tyrosinase preparation as a target protein. Tyrosinase preparation was carried out using Chimera software 1.10.1 that the protein would be separated from the native ligand. The next step was the validation of molecular docking methods. Validation of the molecular docking method was done by inserting the native ligand back into the enzyme that has been removed using the Autodock 4.2 program. The validation parameter of the molecular docking method was the Root Mean Square Distance (RMSD) value ≤ 3.0 Å, which indicated that the accepted protocol and docking the test compound to the target protein could be done (Jain and Nicholls, 2008). The final step was docking of catechin compounds to

tyrosinase by tethering the optimized catechin compound on the prepared target protein. The catechin compound's docking process in the target protein results in a bond energy value and the type of hydrogen bond so that affinity and molecular interaction could be identified, which occur between catechin and the target protein.

Analysis of *in silico* testing

In this study, the energy of the catechin bond was compared with the kojic acid bond energy, which was a positive control that proven as a lightening agent (Laksmiani and Nugraha, 2019). If the energy of the catechin bond was lower than the kojic acid bond energy, then catechins had the potential to be skin lightening agents.

In vitro assay

The principle of the *in vitro* method was based on the presence of dopachrome products, which are the result of L-DOPA oxidation by the tyrosinase enzyme (Zolghadri et al., 2019). Skin whitening compounds will compete with L-DOPA to bind to the tyrosinase enzyme. The competition will reduce the number of dopachrome products produced so that the whitening compound inhibitory activity could be calculated. Dopachrome that were formed with dark orange to red color (Solano, 2014).

Phosphate buffer pH 6.5 was made, followed by making L-DOPA 2.5 g and tyrosinase solution 240 units/mL in phosphate buffer. Fifty mM buffer solution and L-DOPA solution pipetted into the test tube were incubated for 10 minutes. The solution was added with a tyrosinase solution and again incubated for 25 minutes at room temperature and measured the maximum absorption of the dopachrome at 480 nm with a spectrophotometer-UV mini 1240 (Shimadzu, Europe) (Lukitaningsih and Holzgrabe, 2014). The activity of avocado seed extracts containing catechin as a lightening agent was determined by its inhibition of tyrosinase. The non-inhibiting test solution was made with a solution of phosphate buffer and L-DOPA solution and incubated at room temperature for 10 minutes. The solution was added with tyrosinase and then homogenized with a vortex mixer and re-incubated for 25 minutes at room temperature. The non-inhibiting solution was measured for its absorbance at the maximum wavelength (a). Test solutions with inhibitors were made for samples and kojic acid as positive control solutions. Test solutions with inhibitors were made with the same treatment with solutions without inhibitors and added with samples with variations in concentrations of 30 - 150 ppm and 20 - 100 ppm of kojic acid (b).

Analysis of *in vitro* assay

The absorbance value was used to evaluate % inhibition. Equation [1] was the calculation of % tyrosinase inhibition. Percentage of inhibition data were used to determine IC_{50} values by plotting sample and kojic acid concentrations *vs.*% inhibition. The linear equation obtained from the curve was used to predict the IC_{50} value of catechins from avocado seed extracts and kojic acid, which have tyrosinase inhibitory activity of 50% (Laksmiani and Nugraha, 2019).

$$\% \text{ inhibition} = [(a-b)/a] \times 100\% \quad [1]$$

Where a: absorbance of the non-inhibiting solution, and b: absorbance of the test solution (inhibiting solution).

Statistical analysis

Statistical Analysis: Data were given as mean ± standard deviation (SD) of three measurements. The IC_{50} values of extracts as samples, and kojic acid as a standard tyrosinase inhibitor, were calculated by linear regression analysis. All tested samples were statistically analyzed using independent t-test (IBM SPSS Statistics Version 26). P values of less than 0.05 were regarded as statistically significant.

RESULTS

Identification of catechins in extracts

The identification of catechins in avocado seed extract is crucial to ensure that catechins are specific compounds in avocado seeds, and the catechins cause avocado seeds to have therapeutic or pharmacological effects. Also, the study for the ability of avocado seed extract as a skin lightening agent by

in silico could be done with catechins as the active compound of avocado seed extract, by evaluating its inhibitory effect of the tyrosinase enzyme as a target protein. Qualitative and quantitative analysis of the extract obtained was carried out by the TLC-Densitometry method. Qualitative analysis was carried out by looking at the spectrum, and Rf value produced. In contrast, quantitative analysis was carried out using a densitometer instrument by looking at the AUC value so that the levels of catechin compounds in the avocado seed extract can be determined. Standard catechins produce Rf 0.6 and in the sample bottle, there is a spot with Rf 0.6 as well. The same Rf value between standard and sample indicates the presence of the same compound at the migration distance and could be ascertained avocado seed extract containing catechins. In this study, three variations of solvents were used, namely ethyl acetate, ethanol 95%, and acetone. Furthermore, the *in vitro* test uses avocado seed extract with a solvent that produces the highest amount of catechins.

Fig. 1 shows a spectrum of avocado seed extract with various extracting solvents. The spectrum of ethyl acetate and acetone extract of avocado seeds following the standard catechins spectrum, so it was suspected that the extract containing catechins. Whereas the ethanol extract spectrum was not under the standard catechin spectrum

In silico assay

The active compound, catechin and kojic acid that have been prepared and optimized were then docking to the target protein that has been separated from its native ligand using the Autodock Tools 1.5.6 program. The method validation determined a grid box, area or pocket for docking the test compound to the target protein. Table 1 presented the results of docking test compounds with target proteins, and Fig. 2 showed interaction catechin and kojic acid to tyrosinase.

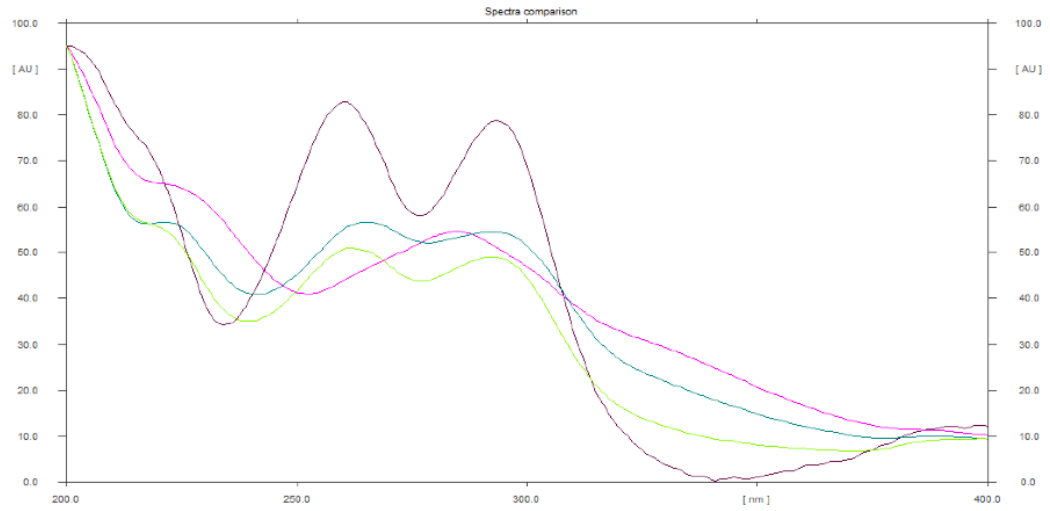

Figure 1. Overlapping spectrum extract of avocado seeds and standard catechins.
standard catechin spectrum; ethanol extract of avocado seeds; ethyl acetate extract of avocado seeds; acetone extract of avocado seeds

Table 1. The energy of catechin and kojic acid bonds to target protein tyrosinase.

Ligand	Bond energy (kkal/mol)	Hydrogen bond	Ligand-protein
Catechin	-7.64	HIS296	O-HE2
Kojic acid	-5.03	HIS259	O-HE2
		HIS296	O-HE2

HIS296: Histidin 296; O-HE: Interaction between oxygen atom from ligand and hydrogen in E2 positions.

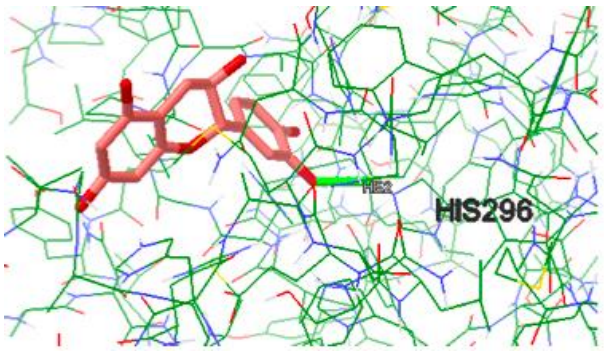

Figure 2. Docking display of the interaction of catechin and kojic acid to the tyrosinase target protein. The 3D interaction between catechin and tyrosinase **(A)**. Interaction between kojic acid and tyrosinase **(B)**.

In vitro assay

The inhibition of tyrosinase was conducted using UV spectrophotometric method by measuring the absorbance of dopachrome. Dopachrome was an oxidation product of L-DOPA that was catalyzed by tyrosinase. Fig. 3 demonstrated the maximum wavelength of dopachrome at 480 nm.

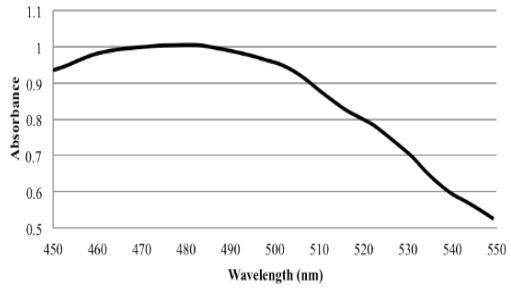

Figure 3. The spectrum of dopachrome from 450-550 nm wavelength.

Fig. 4A exhibits the tyrosinase inhibition activity of kojic acid and Fig. 4B shows the activity of the ethyl acetate extract from avocado seed containing catechin as a skin lightening agent that associated by inhibiting the tyrosinase activity. The regression equation was y = 0.7814 x + 11.973 (R^2 = 0.99523) and the IC_{50} value of kojic acid was 48.67 ± 0.1 µg/mL. Avocado seed extract regression equation obtained was y = 0.5588 x – 1.9795 (R^2 = 0.94283) and the IC_{50} value was 93.02 ± 1.98 µg/mL. The IC_{50} values between the sample and kojic acid presented statistically significant differences ($p<0.05$).

DISCUSSION

All avocado seed extracts with three different types of extracting solvents produced the same Rf as the standard Rf of catechins. However, the avocado seed ethanol extract did not provide the same spectrum as the catechin standards. This phenomenon happened because, in the ethanol structure, there were groups that tend to be polar, and groups tend to be nonpolar. They attracted other components or impurities and affected the spectrum of measurement results. Most catechins were found in ethyl acetate extract of avocado seeds (25.5%), followed by ethanol extract (20.87%) and acetone extract (14.48%). This result could be due to the catechins having four phenol groups whose solubility was limited in polar solvents and one hydroxyl group with solubility in polar solvents (Pauli et al., 2014). This trait brought about by the substituents caused the catechins to be semipolar. Ethyl acetate had a dielectric constant of 6.02, the dielectric constant of acetone was 20.7, and 96% ethanol had a dielectric constant of 24.3. Based on this, it could be seen that ethyl acetate was a solvent with semipolar properties, while acetone and ethanol had features that tend to be polar (Mukesh and Rakesh, 2011). Semipolar properties were those that correspond to catechins so that the catechins in avocado seeds had the highest solubility in ethyl acetate.

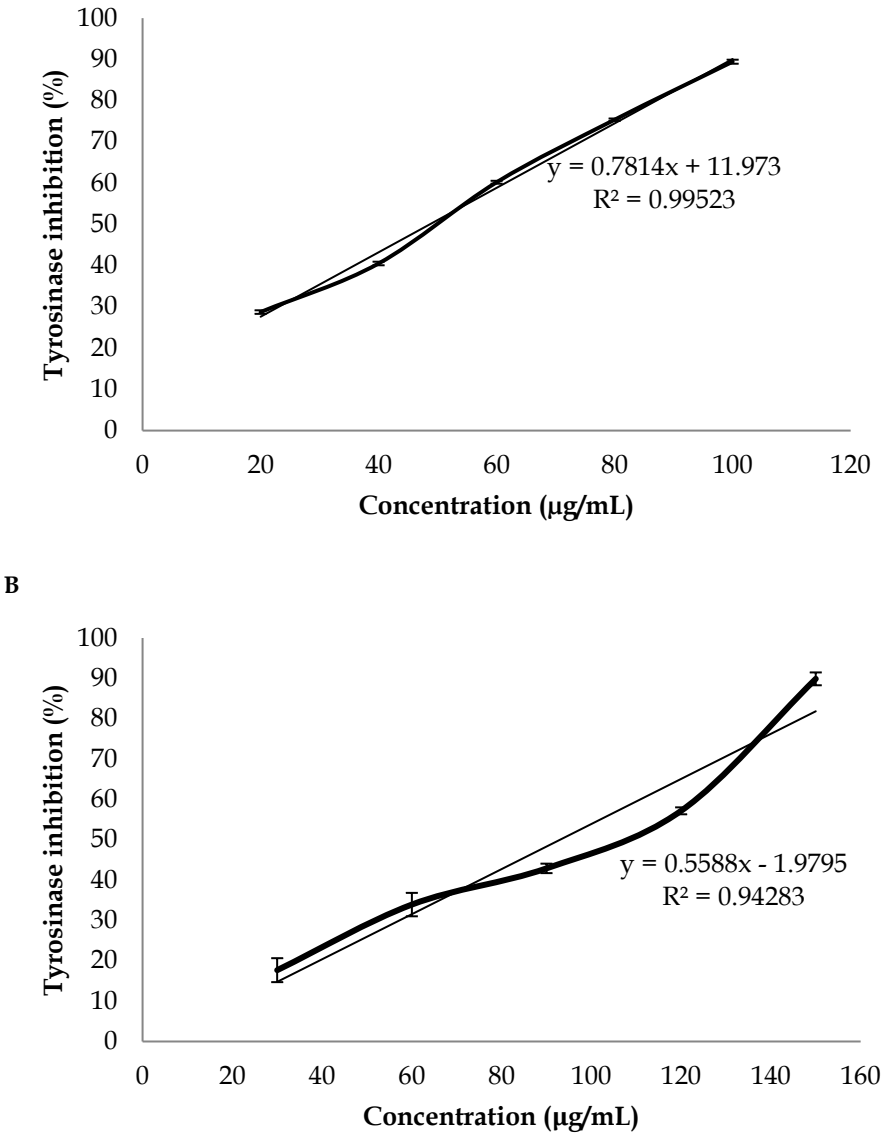

Figure 3. (A) Linear regression curve relation between kojic acid concentration *vs.* its % tyrosinase inhibition and **(B)** ethyl acetate extract of avocado seeds containing catechin concentration *vs.* its % tyrosinase inhibition.

Data were expressed as mean ± SEM (n=3).

In silico research begins with optimization of the 3D structure of catechin and kojic acid. The process of optimizing the composition of catechins in a 3-dimensional structure was carried out with two starts, namely single point calculation then geometry optimization. Geometry optimization was done to minimize the total energy of the composition structure. This process causes the structure to become more stable (Mukesh and Rakesh, 2011). The total energy of the single point calculation and geometry optimization obtained from catechin compounds are -3861.98 kcal/mol and -3885.12 kcal/mol respectively, while the total energy of the single point calculation and geometry optimization derived from successive kojic acid according to -1694.92 kcal/mol and -1699.67 kcal/mol. Based on the total energy obtained, it could be stated that catechins and kojic acids have been successfully optimized, which were characterized by the total energy value of the results of geometry optimization lower than the total energy of single-point calculations. The lower bond energy of a compound means the compound would be more stable because

higher energy was needed to interfere with the bonds.

Tyrosinase preparation in this study was carried out using the Chimera 1.11.1 program. The preparation of target proteins begins with the selection of a chain of target proteins containing native ligands, then the separation of native ligands from the selected target protein chains. The chosen tyrosinase chain was chain A. *In silico* method could be done if it had fulfilled the validation requirements that had been set. Validation obtained ten conformations, and one conformation was chosen with the lowest RMSD value (Jain and Nicholls, 2008). The validation of the molecular docking method of catechins and kojic acid on the tyrosinase indicated that the RMSD value was ≤ 3 Å (2.16 Å), which states that the method used has met its validation requirements.

Table 1 showed that catechin compounds had a lower bond energy value than kojic acid against the target protein of tyrosinase. It can be stated that catechins had enormous potential in inhibiting the tyrosinase through *in silico* study. The lower the ligand bond energy with the target protein, the stronger the bond formed between the ligand with the target protein, and so does the affinity of the ligand against the target protein, which was directly proportional to the strength and stability of the bond.

The determination of catechins extract activity depended on the ability of the compounds to inhibit tyrosinase, so the product of tyrosinase was not formed. The number of products created was measured according to the sample absorbance measured at the maximum wavelength of dopachrome. Dopachrome was a natural polymerization product from dopaquinone in the process of forming melanin (Solano, 2014). Based on the regression curve of Fig. 3, it was known that the maximum wavelength of dopachrome was 480 nm, so the measurement of the sample was done at that wavelength.

The IC_{50} value of ethyl acetate extract of avocado seeds was higher than kojic acid and statistically significantly different ($p<0.05$). However, the IC_{50} amount of the extract was less than 100 µg/mL (Lee et al., 2010), which means that the extract had vigorous activity as a tyrosinase inhibitor agent. The avocado seed extracts were very promising to be developed into a skin lightening agent.

CONCLUSIONS

Avocado (*Persea americana* Mill.) seed extracts containing catechins have a potential activity as skin lightening agents through inhibiting the tyrosinase. In this research, the catechin content in the extracts was not pure. For this reason, further research needs to be done using purified fractions of ethyl acetate extract to get an increased activity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Laksmiani NPLL	Sanjaya IKN	Leliqia NPE
Concepts or ideas	x	x	x
Design	x	x	x
Definition of intellectual content	x	x	x
Literature search		x	
Experimental studies	x	x	
Data acquisition	x	x	x
Data analysis	x	x	x
Statistical analysis			x
Manuscript preparation	x	x	x
Manuscript editing	x	x	x
Manuscript review	x	x	x

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