ISSN 0974-3618 (Print)
0974-360X (Online)
www.rjptonline.org

RESEARCH ARTICLE

Antioxidant Activity and Caffeine content of Cascara from *Coffea canephora* Var. *Robusta* in Pangandaran West Java: A Study for Potential of Antihyperpigmentation

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ABSTRACT:
Cascara is a very abundant by-product of coffee. Coffee cascara contains antioxidant compounds. Antioxidant compounds can protect the skin from sun exposure and free radicals which can cause increased melanin production resulting in hyperpigmentation. Although there has been no specific research on antihyperpigmentation cascara extract from *Robusta coffea*, several studies have shown that antioxidant compounds and inhibitor tyrosinase can help reduce hyperpigmentation on the skin. The current study investigates the antioxidant capacities of cascara from *Robusta coffea*, its phytochemical profile, caffeine content, and examines the potential interaction of caffeine with the tyrosinase in human skin using an insilico approach. **Methods:** The extraction process was carried out using the Soxhlet method with a sequential increase in solvents (n-hexane, ethyl acetate, and ethanol). The extract obtained was evaluated for antioxidant activity by DPPH and CUPRAC methods, preliminary phytochemical screening, and total phenolic content (TPC). Caffeine content was measured by HPLC. The potential of caffeine in the inhibition of tyrosinase enzyme was evaluated by molecular docking with kojic acid as the standard of inhibitor tyrosinase. **Result:** Based on the Antioxidant Activity Index (AAI) DPPH and CUPRAC, cascara extract from *Robusta coffea* had moderate to strong antioxidant categories. Phytochemical screening of n-hexane extract revealed the presence of alkaloids, quinones, and steroid/triterpenoids while in ethanol and ethyl acetate extract revealed the presence of alkaloid, phenol group, flavonoid, quinones, and steroid/triterpenoid. TPC in cascara extract from Robusta was 2.334 to 10.44 g GAE/100 g extract. Caffeine content by HPLC in ethanol extract was 0.6613% ± 0.007. The interaction of kojic acid and caffeine with tyrosinase enzyme was -5.7 and -5.8 kcal/mol. **Conclusion:** Ethanol cascara extract from *Robusta coffea* has more potential for anti-hyperpigmentation through the inhibition of free radicals by antioxidant compounds and the inhibition of tyrosinase. This is worthy of further investigation in the future.

KEYWORDS: Cascara, Robusta, Caffeine, Antihyperpigmentation.

Received on 04.07.2024 Revised on 11.01.2025
Accepted on 07.05.2025 Published on 02.08.2025
Available online from August 08, 2025
Research J. Pharmacy and Technology. 2025;18(8):3557-3564.
DOI: 10.52711/0974-360X.2025.00512

INTRODUCTION:

Reactive Oxidative Singlets (ROS) can be produced when exposed to UV radiation and can cause aging of the skin[1]. Hyperpigmentation is one form of manifestation of aging. The condition known as skin hyperpigmentation causes certain areas of skin to darken more than the surrounding normal skin tone. This happens when the skin's melanin production is excessive in some areas[2]. Under normal physiological conditions, melanin is generated and serves as a ROS scavenger and

photoprotectant. However, dermatological issues including freckles, solar lentigo (age spots), and melasma are linked to an overproduction of melanin as a result of prolonged skin exposure to UV radiation[3]. Tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2) lead to the synthesis of melanin[4]. Therefore, antioxidant and tyrosinase inhibitors are essential in the prevention and treatment of hyperpigmentation.

Coffee is a highly consumed product noted for its distinct flavor and scent throughout the world[5]. Cascara, mucilage, silverskin, and parchment are examples of coffee by-products[6]. Cascara, mucilage, and parchment produced 39-45kg/100 kg, 22kg/100kg, and 39kg/100 kg of cherry, while silverskin produced 2.08kg/100kg of bean[7]. Bioactive chemicals are found in coffee by-products and have potential applications in a variety of industries, including food, medicine, and cosmetics[8]. Cascara from *Robusta coffea* was reported to have biological activity as an antioxidant and antibacterial. Coffee by-products are widely used in cosmetic formulations example as emollient, antioxidant and anti-aging, photo protectors, and anti-cellulite[9]. This shows that coffee by-product has the potential to be investigated and developed into medicinal raw materials and cosmetic raw materials.

Phenol and alkaloids are the two major groups of metabolites in cascara coffee. Phenolic compounds can play a role in the inhibition of tyrosinase enzyme[10]. Caffeine is the compound of alkaloid group metabolite in cascara coffee. An increasing amount of study over the years has suggested that caffeine may have antioxidant properties due to its capacity to scavenge free radical[11]. Besides that, caffeine has been widely used for a variety of health problems such as cardiovascular, neuroprotective, liver cancer, and skin cancer[12]. As a utilization in the field of cosmetics has been widely used as a photoprotective[13]. Research on caffeine as a tyrosinase inhibitor has been sparsely reported. In silico, caffeine has an affinity of (-2.86 kcal/mol) towards tyrosinase enzyme derived from mushrooms[14]. For the present, our study is to determine how strong the affinity of caffeine is towards the tyrosinase enzyme present in human skin.

MATERIALS AND METHODS:

Materials:

The materials used included DPPH (2,2-diphenyl-1 pichydrazyl) (Sigma-Aldrich), ascorbic acid (Sigma-Aldrich), Gallic acid (Sigma-Aldrich), ethanol 96%, methanol, ethyl acetate, n-hexane, and other analytical grade reagents obtained from Merck.

Sample Preparation:

Cascara (pulp and skin) *Robusta coffea* from wet method processing was collected from a local gardener in Pangandaran, West Java in April 2022. Subsequently, the fresh coffee cascara produced from de-pulping cherry coffee was washed, dried at 40ºC–45ºC, and ground into powder.

Methods of extraction:

To obtain the extract cascara from *Robusta coffea*, the Soxhlet method with an increasing solvent polarity was used. The first, 300g of powder cascara pulp was xtracted using n-hexane, while the filtrate and residue were separated. After the residue was dried, ethyl acetate was used for the extraction process. Finally, ethanol was used to extract the residue[1]. An evaporator that rotated at 50°C was used to thicken each extract. Three separate runs of the Soxhlet extraction procedure were performed.

Antioxidant Activity:

a) DPPH method :

Antioxidant capacity was adopted in Blois's method using a UV-Vis spectrophotometer (Beckman Coulter DU 720) and absorbance measurement at λ 517nm. The free radical DPPH with a concentration of 0.1mM or 39.4µg/mL in methanol pro analysis was mixed with various concentrations of extract (Volume 1: 1) and incubated for 30min[15]. The standard was ascorbic acid and a three-replication analysis was conducted.

For calculating the percentage of free radical inhibition was used the formula :

$$\% \text{ inhibition} = \frac{\text{Blank abs} - \text{Sample abs}}{\text{Blank abs}} \times 100$$

To determine the IC_{50}, a calibration curve was created between the percentage of DPPH scavenging activity and the concentration.

b) CUPRAC method:

Antioxidant capacity with CUPRAC was used by Apak[16] method and Hartati[17]. $CuCl_2$ in aquadest and neocuproine in ethanol were combined to create the 100 µg/mL solution, which was dissolved in ammonium acetate buffer pH 7. Several extracts were mixed into the CUPRAC solution (volume 1:1) with an incubation time of 30minutes and the standard antioxidant compound was used ascorbic acid. After incubation, absorbance was measured at a wavelength of 450nm and the procedure was repeated three times. From the calibration curve, the exhibitory concentration of 50% (EC_{50}) was determined.

Antioxidant Activity Index:
Antioxidant activity index (AAI) was the last measurement used for evaluating antioxidant activity. It was determined using the formula AAI = (Final concentration of DPPH [µg/ml])/IC_{50} (µg/ml)[18].

Phytochemical Analysis:
Phytochemical screening was adopted in Fitriansyah[15] and used to identify alkaloids, flavonoids, tannins, triterpenoids/steroids, quinones, and saponins to evaluate the presence of secondary metabolites in extract cascara from *Robusta coffea*.

Determination of Total Phenolic Content (TPC):
TPC was calculated by modifying Pourmorad's methods[19]. Three separate extracts were tested for each. Furthermore, the measurement wavelength for the test was 765nm. Gallic acid equivalent (GAE) per 100g extract (g GAE/100g extract) was adopted to express TPC, which was determined using a calibration curve with concentrations ranging from 10 to 250µg/ml.

HPLC Analysis for Caffeine:
One of the most often utilized analytical methods for figuring out caffeine is high-performance liquid chromatography (HPLC)[20,21] and UV spectroscopy coupled with multivariate analysis[22]. The cascara extract was analyzed for its caffeine based on chromatogram HPLC (Arc™ HPLC with PDA Detector. The analysis was carried out with coloum used Reliant ® T3 C18 5 µm (4.6 x 150mm) in 40°C. The mobile phase was prepared by mixing A (Water) and B (Acetonitrile+ 0.1% Formic Acid). The retention was made 25 minutes, with scheme 0.0-7.0 minutes (14-15%B), 7.0-15.0 minutes (15-20%B), 15.0-15.1 (20-100%B), 15.1-20.0 minute (100%B), 20.0-20.1 minute (14%B), 20.1-25.0 (14%B). The flow rate used was 0.8 ml/min and set at 272nm[23]. The concentration of caffeine in extract measurement by standard caffeine calibration curve.

Molecular Docking:
a) Protein modeling of hTYR:
The protein modeling of hTYR was performed prior to Kumari[24]. A three-dimensional structural model of hTYR was built using homology modeling with the MODELLER program[25]. The sequence of the tyrosinase protein (Homo sapiens) was retrieved from UniProt (P14679.1)[26]. The template structure used in this model was the same as the previous one, selected based on the highest similarity from a BLASTp search. The PDB ID 5M8Q, which showed a maximum of 44.67% identity and 61% similarity, was used as the template. The crystal structure of human tyrosinase-related protein 1 (TYRP1) was determined using X-ray diffraction with a resolution of 2.85 Å, an R-value free of 0.275, and an R-value work of 0.208[27].

Modeling was performed by removing 13 amino acids from the start along with a short sequence of 73 residues from positions 456 to 529 of the peptide. Both sequences were aligned using structural alignment with the T-Coffee server (Expresso)[28]. Two copper ions and a water molecule were added to the catalytic center, obtained from bacterial tyrosinase (PDB ID 3NM8), by overlaying both structures[29].

The overall quality of the modeled structure was assessed through the DOPE score and GA341. The model structure was evaluated using the Ramachandran plot with PROCHECK and the Z-score from ProSA-WEB[30].

b) Ligands and receptor structure preparation:
Caffeine structure was retrieved from PubChem (CID 2519), while kojic acid was extracted from PDB ID 5M8Q to investigate their interaction with modeled hTYR. The 3D structure of caffeine was downloaded in sdf format. All structures (caffeine, kojic acid, and hTYR) were converted into pdbqt file format by adding the atom type and Gasteiger charges using AutoDockTools-1.5.6.

c) Active site prediction and molecular docking studies:
Active site prediction was performed by CastP web server[31]. Docking of all ligands was performed with AutoDock Vina using 8 exhaustive processors. Ligand molecule-docking was performed on a predefined grid obtained from the template structure in complex with kojic acid[27], along with the active site prediction. The grid box was sized 22Å × 22Å × 22Å, with dimensions for X, Y, and Z coordinates being -15.191, -2.818, and -25.978, respectively. Docking validation was achieved by selecting the best possible conformation of each ligand based on the lowest docking score and their binding interactions measured in terms of Gibbs free energy (ΔG). The molecular interactions were analyzed and evaluated using BIOVIA Discovery Studio Visualizer 2017.

RESULT:

Antioxidant activity:
Antioxidant activity in ethanol extract cascara *Robusta coffea* based on DPPH and CUPRAC assay. Total phenolic content and caffeine content are very important information in antioxidant activity. Antioxidant activity is represented in IC_{50} and classified using the value of Antioxidant Activity Index (AAI) (Table 1.). The concentration of the sample or standard that can raise the 50% absorbance of CUPRAC capability is known as the EC_{50} of compounds. The concentration of the sample or standard capable of lowering 50% of the absorbance of DPPH is the IC_{50} of DPPH scavenging capacity. The

best capacity antioxidants also had the lowest IC50 or EC50 values. The IC_{50} results may differ depending on the concentration of the radical solution used. However, when we proceed to the AAI value it will show the same potential group[17].

Table 1. IC_{50} of cascara pulp extract from robusta coffee

Sample	IC_{50} DPPH	EC_{50} CUPRAC	AAI DPPH	Aai Cuprac
n-hexane	70.799 ± 0.228	227.569 ± 2.375	0.557± 0.008	0.219± 0.002
Ethyl acetate	20.176 ± 0.292	37.403 ± 0.422	1.953± 0.028	1.337± 0.015
Ethanol	10.500 ± 0.455	91.976 ± 0.552	3.752± 0.159	0.544± 0.003
Ascorbic acid	6.020 ± 0.025	20.36 ± 0.06	2.458± 0.241	2.465± 0.315

Phytochemical Screening:

Phytochemical screening was carried out on crude drugs and ethanol, ethyl acetate, and n-hexane extract of cascara from *Robusta coffea*, shown in Table 2.

Table 2. Phytochemical screening of crude and extract cascara pulp of Robusta coffee

Cascara pulp Robusta	Phytochemical screening					
	Alk	Tan	Fla	Sap	Qui	Ste
CD	+	+	+	-	+	+
RCN	+	-	-	-	+	+
RCA	+	+	+	-	+	+
RCE	+	+	+	-	+	+

Alk-Alkaloid; Tan-Tannin; Fla- Flavonoid; Sap- Saponin; Qui- Quinone; Ste- Steroid/Triterpenoid
CD = Crude drugs; RCN = n-hexane Robusta cascara extract; RCA = ethyl acetate Robusta cascara extract; RCE = ethanol Robusta cascara extract; (+) = detected; (-) = not detected

Total Phenolic Content:

TPC is equivalent to gallic acid in extract. According to the result, TPC in ethanol cascara extract from *Robusta coffea* was higher than n-hexane and ethyl acetate.

Table 3. TPC of cascara pulp extract from robusta coffee

Sample	Yield %	TPC (g GAE/100 g extract)
n-hexane	1.34%	2.334 ± 0.231
Ethyl acetate	1.15%	7.899 ± 0.421
Ethanol	3.12%	10.44 ± 1.243

HPLC Analysis Caffeine in Ethanol Extract:

Detected caffeine and quantification of caffeine content in the extract was performed by an HPLC instrument with C18 column, at the wavelength 274 nm. Fig 1. shows the caffeine standard and in Fig.2 shows general extract chromatograms. The caffeine content in the extract has 0.6613% ± 0.0077.

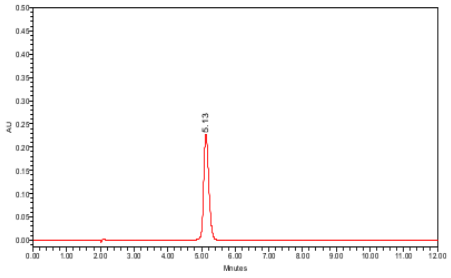


Figure 1. HPLC chromatogram of caffeine standard

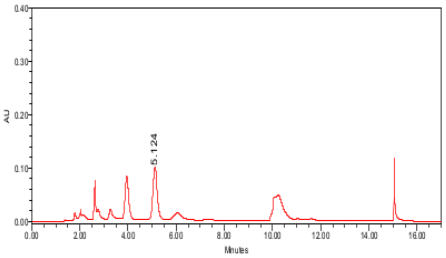


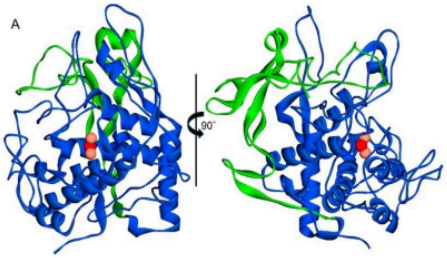
Figure 2. HPLC Chromatogram of caffeine in ethanol extract cascara from *Robusta coffea*

Molecular docking caffeine:

a) Protein modeling:

The alignment of the template and model is a crucial step in protein modeling[25], thus to avoid misalignment, a structural alignment was performed. Although the identity of the template was less than 50%, the alignment showed that both structures were similar, with a score of 99.

The Ramachandran plot and ProSA were used to evaluate the quality of the hTYR model (Figure 3). Ramachandran plot analysis indicated that more than 90% of amino acid residues were in the allowed region. Furthermore, the z-score analysis provided an overall quality assessment of the model in the X-ray region, with a score of -8.18[32]



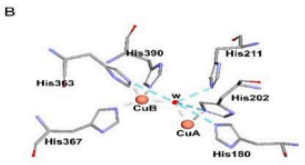


Figure 3. The model structure of hTYR. A. The Cys-rich and active site of hTYR are presented in green and blue ribbon, respectively. While the Cu ions and a water molecule are depicted in pink and red ball. B. The amino acid located in the surounding of Cu ions and a water molecule

b) Molecular docking studies:

Active site prediction by CastP resulted in one active site with a total area of 483.059 Å² and a total volume of 488.712 Å³, respectively (Figure 4). The docking results for kojic acid and caffeine are presented in Figure 5, showing similar orientations and binding energies of -5.7 and -5.8 kcal/mol. The intramolecular interactions between the ligands and the receptor are presented in (Figure 6).

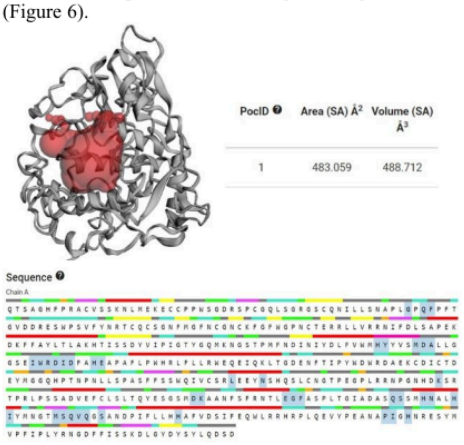


Figure 4. Active site prediction by CastP.

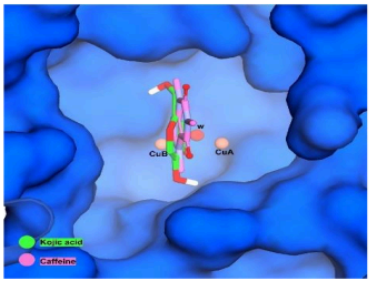


Figure 5. Molecular docking results of kojic acid (green) and caffeine (pink) located in the active site prediction.

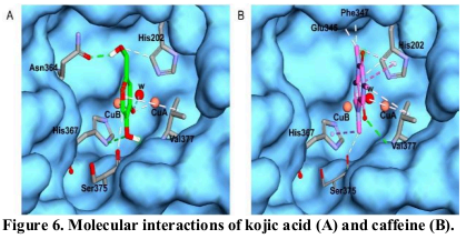


Figure 6. Molecular interactions of kojic acid (A) and caffeine (B).

DISCUSSION:

In this study, we used a variety of assays to map and understand the antioxidant potential of cascara extract from *Robusta coffea*. Antioxidant is classified into main or secondary types based on their functional mechanisms. These compounds operate as hydrogen donors or acceptors of free radicals to interrupt the oxidation chain reaction, generating more stable radicals. The DPPH (2,2-diphenyl-1-picrylhydrazyl) method serves as a valuable tool for evaluating the antioxidant activity of coffee extract. This method gauges the ability of the extract to inhibit the free radical DPPH. Furthermore, this radical, which is insoluble in water but soluble in various organic solvents, dissolves in aqueous solutions of methanol, ethanol, or a combination of both[33]. In the early 2000s, the CUPRAC test was developed as a method for measuring total antioxidant capacity. This method has been applied to matrices containing both hydrophilic and lipophilic antioxidant[33]. The antioxidant activity of the extract reflects the differences in the species, chemical composition, and solvent. In this study, extraction was carried out in stages with increasing polarity of the solvent, namely n-hexane, ethyl acetate, and ethanol. N-hexane is a nonpolar solvent that preferentially extracts only nonpolar molecules, hence extract exclusively contains nonpolar chemicals. The majority of the semipolar chemicals and a tiny amount of nonpolar compounds are extracted using ethyl acetate[17]. Finally, ethanol is used for extraction, which is a solvent predominantly used to extract polar molecules as well as tiny amounts of nonpolar and semipolar chemicals.

The current study found that IC_{50} of DPPH from ethanol extract was lower than n-hexane and ethyl acetate extract. Meanwhile, the IC_{50} of CUPRAC from ethyl acetate was lower than n-hexane and ethanol extract. Antioxidant activity was divided into four classified based on AAI, namely weak (AAI < 0.5), moderate (0.5 ≤AAI < 1), strong (1 ≤AAI≤ 2), and very strong (AAI > 2)15. The result showed cascara extract from *Robusta coffea* had moderate to strong antioxidant categories. However in general, ascorbic acid more a higher potent antioxidant properties than extract cascara from *Robusta coffea*. The aqueous extract Robusta cascara from the

wet method has antioxidant activity of 33.5% at 100 ppm which means an IC_{50} above 10ppm[34]. Ethanol extract of Robusta cascara pulp has IC_{50} to DPPH was 12.75µg/mL[35]. Until the present, there have been few reports on the antioxidant activity of robusta coffee cascara.

Cascara extract from *Robusta coffea* extract contained alkaloid, flavonoid, tannin, quinone, and steroid/triterpenoid group metabolite. Although it is not yet known how many contents, this information shows whether there are metabolites that can contribute to antioxidant activity and tyrosinase inhibition. According to the result, TPC in ethanol cascara extract from *Robusta coffea* was higher than n-hexane and ethyl acetate. The TPC of ethanol extract from Robusta coffee cascara pulp was 1.85 mg GAE/100 mg measured by the Folin-Ciocalteu method[34]. TPC in Robusta cascara pulp extract, characterized by different drying physical properties, varied within the range of 2.98mg GAE/g extract to 14.36 mg GAE/g extract[36]. In a separate study, the TPC of Robusta coffee cascara pulp extract from Aceh as 80.00 mg GAE/100 g extract[37].

TPC values of the samples showed the quantity and variety of phenolic compounds, accountable for a range of biological functions and antioxidant characteristics. Many variables impacting TPC include the kind of plant, the type, the extraction method, and the solvent used solvent used[38].

Based on HPLC chromatogram of caffeine standard showed caffeine detected at rt 5.13. Based on the HPLC chromatogram of the extract, caffeine was detected at rt 5.124min. The concentration of caffeine in the extract was calculated using the calibration curve of standard caffeine with regression linear equation y = 19572 – 53262, R^2 = 0.9999. The caffeine content in coffee can vary depending on the variety of coffee[39]. Phenolic acid, ferulic acid, caffeine, caffeic acid, and chlorogenic acids were identified in fresh coffee cascara[40], and contributing to antioxidant activity. Caffeine is one of the bioactive substances in coffee. It was 1% caffeine content in green bean Robusta[41]. The caffeine content in coffee husk from *Robusta coffea* was 1.1% with supercritical extraction[42]. Meanwhile in Robusta green coffea beans were 1.82 mg/g of coffee[43]. The other study, showed that the caffeine content in Robusta bean was 18,64 mg/g[44]. So far, the results of research on the caffeine content of cascara extract from *Robusta coffea* have not been widely reported.

Robusta coffee bean extract has active compounds as anti-hyperpigmentation which can be role play by increasing the tyrosinase enzyme. These compounds are chlorogenic acid and caffeine[45]. In Elfiah's study, it was concluded that 5% Robusta coffee bean extract can be considered as one of the topical therapies for hyperpigmentation problems[46]. In our research, based on a molecular docking study, caffeine can inhibit tyrosinase enzyme in human skin. The first step in molecular docking study, protein modeling needs to be done. In protein modeling, the model constructed from homology modeling needs to be assessed to prevent deviations from the wild-type conformation. The model's evaluation was conducted using the DOPE and GA341 values. The lowest DOPE score for hTYR was -51002, and the GA341 value was 1. A lower DOPE score indicates a more stable model, while a GA341 value greater than 0.7 represents a good-quality folding pattern. From Ramachandran plot analysis and the z-score analysis, We may assume that the structure of our model is of high quality. Since tyrosinase activity depends on the presence of two copper ions and a water molecule, they were added to the catalytic center from bacterial tyrosinase, surrounded by histidine residues (Figure 3)[27].

Active site prediction by CastP resulted in one active site with a total area of 483.059 $Å^2$ and a total volume of 488.712 $Å^3$, respectively (Figure 4). This active site also corresponds to the binding site of kojic acid found in the template structure. The docking of kojic acid and caffeine was successfully achieved with AutoDock Vina[47,48] and analyzed in terms of binding energy. Hydrogen bonds (both conventional and carbon) and hydrophobic interactions contributed to binding both ligands to the receptor. Both copper ions and the water molecule also contributed to ligand binding, especially with kojic acid. This binding pattern is similar to that observed in TYRP1[27].

Hydrogen bonds are crucial for the specificity and strength of ligand binding. Kojic acid forms more hydrogen bonds than caffeine, but the specific residues involved and their impact on binding affinity vary. Caffeine has a slightly better docking score than kojic acid, suggesting it binds marginally stronger to tyrosinase. Caffeine forms more non-bond interactions compared to kojic acid, which could contribute to its slightly better docking score. Caffeine also has more hydrophobic interactions, which are essential for the stability of the ligand-protein complex in the protein's hydrophobic core. Both ligands showed comparable values and analysis, indicating that caffeine and kojic acid have similar inhibitory abilities. Some amino acids contributing to the binding of kojic acid and caffeine are His202, His367, Ser375, and Val377. These residues might play essential roles in enzymes.

CONCLUSION:

The extract cascara from *Robusta coffea* has moderate to strong antioxidant activity. The ethanol extract cascara had strong antioxidant activity. Molecular docking studies reveal the potential of strong interaction between the caffeine in ethanol extract cascara from *Robusta coffea* with human tyrosinase enzyme. Caffeine has similar inhibitory abilities with kojic acid as standard inhibitors of tyrosinase. This data can be information to consider the ethanol extract of cascara from *Robusta coffea* as an ingredient for further research as an anti-hyperpigmentation.To verify the anti-hyperpigmentation properties of the caffeine and other phytometabolite found in cascara from *Robusta Coffea*, more studies using in vitro and in vivo designs are necessary.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation.

ACKNOWLEDGMENTS:

The authors would like to thank STFI-Hazanah Foundation Internal Research Grants for their kind support of this study.

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