PHYTOCHEMICAL PROFILING AND IN SILICO SCREENING OF BIOACTIVE COMPOUND FROM *Persea americana* MILL. AS CYCLOOXYGENASE-2 INHIBITORS

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Abstract: Pharmacological information on avocado leaves has been studied through phytochemistry, Lipinski, ADMET, protein ligand interactions, molecular docking, density functional theory (DFT), and molecular dynamics. This study aims to determine the secondary metabolite content of avocado leaf extracts using phytochemical screening methods and to assess the ability of bioactive compounds to inhibit cyclooxygenase-2 through molecular docking calculations using the PDB ID 5IKR. Phytochemical screening showed that the ethanol extract contained alkaloids, saponins, tannins, steroids, and flavonoids, while the ethyl acetate fraction lacked saponins. In addition, most of the compounds met the Lipinski and ADMET rules. Avocadene and hippuric acid are potential candidate agents with binding energies of -5.73 kcal/mol and -5.41 kcal/mol, respectively. The binding interaction of avocadene and hippuric acid with the COX-2 receptor is reinforced by hydrogen bonds with MET490 and TYR353. Avocadene exhibits a higher HOMO-LUMO energy gap (7.146 eV) than hippuric acid (5.176 eV). Molecular dynamics simulations confirmed the stability of both complexes, with RMSD values of 1.769 and 1.791 Å, respectively, and showed no significant fluctuations throughout the 100 ns simulation. This study provides a foundation for the development of natural-product-based drugs for inflammatory diseases and requires future in vitro and in vivo validation.
Keywords: avocado; anti-inflammatory agent; phytochemical; molecular docking; molecular dynamics

Abstrak: Informasi farmakologi pada daun alpukat telah diteliti melalui fitokimia, Lipinski, ADMET, interaksi protein ligand, molekuler docking, density functional theory (DFT) dan dinamika molekuler. Penelitian ini bertujuan untuk menentukan kandungan metabolit sekunder pada ekstrak daun alpukat menggunakan metode skrining fitokimia serta mengevaluasi kemampuan senyawa bioaktif dalam menghambat siklooksigenase-2 melalui perhitungan docking molekuler menggunakan PDB ID 5IKR. Penyaringan fitokimia menunjukkan bahwa ekstrak etanol mengandung alkaloid, saponin, tanin, steroid, dan flavonoid, sedangkan fraksi etil asetat tidak mengandung saponin. Selain itu, sebagian besar senyawa memenuhi aturan Lipinski dan ADMET. Avocadene dan hippuric acid sebagai kandidat agen potensial dengan energi ikatan masing-masing sebesar -5,73 kcal/mol dan -5,41 kcal/mol. Interaksi ikatan avocadene dan asam hippurat dengan reseptor COX-2 diperkuat oleh ikatan hidrogen dengan MET490 dan TYR353. Avocadene menunjukkan celah energi HOMO-LUMO yang lebih tinggi (7,146 eV) dibandingkan hippuric acid (5,176 eV). Simulasi dinamika molekuler mengonfirmasi stabilitas kedua kompleks tersebut,

dengan nilai RMSD masing-masing sebesar 1,769 dan 1,791 Å, serta tidak menunjukkan fluktuasi yang signifikan selama simulasi 100 ns. Penelitian ini memberikan landasan untuk pengembangan obat berbasis produk alami untuk penyakit inflamasi dan membutuhkan validasi in vitro dan in vivo di masa mendatang.

Kata kunci: alpukat; agen anti-inflamasi; fitokimia; *molecular docking*; *molecular dynamics*

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Introduction

Inflammation is the body's natural response to various stimuli, such as pain, irritation, infection, and injury, characterized by symptoms like redness, swelling, and bruising. This inflammatory reaction is a non-specific immune response that helps the body combat harmful agents and initiate healing (Reis et al., 2020). However, chronic or uncontrolled inflammation can contribute to the development of various health conditions, including tumor and cancer (Utami et al., 2020). The Cyclooxygenase-2 (COX2) enzyme plays a crucial role in the inflammatory response, making it a primary target for anti-inflammatory treatments. This enzyme catalyzes the conversion of arachidonic acid into pro-inflammatory prostaglandins, which play a role in vasodilation, mediate pain, and edema. Thus, targeting COX-2 provides an effective approach to controlling inflammatory responses (Revankar et al., 2023). Nonsteroidal anti-inflammatory drugs (NSAIDs) have traditionally been the go-to solution for managing inflammation. While effective, long-term use of these synthetic drugs can lead to a range of side effects, including kidney infections, bleeding, and stomach irritation (Sohail et al., 2023). Recent studies indicate that natural compounds remain an important source of potential anti-inflammatory drugs due to their ability to target various mechanisms and their potentially superior safety profile compared with traditional therapies (Choo et al., 2025). Further research into using plant-derived compounds as anti-inflammatory agents could pave the way for developing safer and more effective therapeutic options.

Indonesia's diverse biodiversity provides a wealth of food and medicinal resources. One such plant is the avocado (*Persea americana* Mill.), which is traditionally used for various health benefits. In 2019, Jambi province in Indonesia produced 46,632 (kw) of avocados (BPS, 2019). Research has shown that all parts of the avocado plant possess medicinal potential (Louis et al., 2020). The medicinal application of avocados has been reported across a diverse array of maladies, most notably in the management of skin conditions, diabetes, bronchitis, and diarrheal diseases. Avocado leaves, in particular, are commonly used to treat conditions like hypertension, heart disease, stroke, and kidney failure (Wulandari et al., 2024). The phytochemical composition and phenolic content of avocado plants can be influenced

by geographical factors (Pradani et al., 2024). Studies have revealed that avocado leaf extract contains a variety of secondary metabolites, including alkaloids, saponins, tannins, steroids, terpenoids, and flavonoids (Setyowati, 2022).

Avocado leaves (*Persea Americana* Mill.) are a rich source of secondary metabolites with promising medicinal potential. The secondary metabolites have demonstrated anti-inflammatory properties, as evidenced by research conducted by Suandewi, (2020). Exploring medicinal plants such as avocado leaves offers an attractive alternative to address these concerns. Utilizing the natural bioactive compounds found in these plants could facilitate the design and development of new drugs that exhibit good pharmacological effects while minimizing adverse side effects (Astika et al., 2022). The comprehensive phytochemical profile and documented anti-inflammatory properties of avocado leaves make them a compelling candidate for further investigation as a source of natural, safe, and effective therapeutic agents.

In a recent in vitro study, Ovalle-Marin et al. (2020) demonstrated that avocado leaf extracts possess potent anti-inflammatory properties. Hippuric acid, a compound found in avocado and its derivatives, has attracted attention due to their analgesic, antioxidant, and anti-inflammatory properties (Munawar et al., 2024). The current research aims to comprehensively evaluate the potential of avocado leaves as a source of anti-inflammatory drugs. The study will employ a multifaceted approach, including phytochemical screening, Lipinski's rule of five assessment, ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) predictions, protein ligand interactions, molecular docking calculations, density functional theory (DFT), and molecular dynamics. This integration has recently attracted significant interest in drug research based on natural products, enabling a more in-depth assessment of binding affinity, electronic characteristics and the stability of complexes prior to experimental validation (Alhashemi et al., 2025). This research can serve as a valuable reference for future in vitro and in vivo studies, helping to minimize the risk of poor pharmacological outcomes. The result could provide a promising alternative to synthetic drugs, which are often associated with undesirable side effects. Although the anti-inflammatory activity of avocado leaves has been widely reported, the molecular interactions between their bioactive compounds and COX-2 remain insufficiently characterized. Therefore, investigating their binding mode, electronic properties, and complex stability may provide valuable insights into their anti-inflammatory potential, supporting the development of safer, targeted therapeutic agents.

Method

Material

The tools used are a water bath, analytical balance, test tube, beaker, measuring cup, filter paper, separating funnel, and dropper pipette. The hardware used in this research is a computer with Intel ® Celeron N4020 CPU processor specifications, installed RAM 8.00 GB, system type 64-bit operating system x64-based processor, Windows specifications edition Windows 11 home single language, and

several computational chemistry applications. The materials used were avocado leaves, 96% ethanol, distilled water, $FeCl_3$, HCl, Mg powder, Liebermann-Buchard reagent, Dragendroff's reagent, Mayer's reagent, and Wagner's reagent. Several databases, namely RCSB and Pubchem, were used to obtain information on in silico studies.

Preparation

Avocado leaf samples were washed to remove impurities, then dried and ground into a powder to facilitate extraction of the compounds by the solvent. The small particle size increases the contact surface area between the solid and the solvent (Miftahurrahmah et al., 2021). A total of 100 grams of simplisia was macerated in 500 mL of 96% ethanol for 3 x 24 hours, then filtered to obtain the filtrate and evaporated at 70°C using a water bath. The concentrated extract was redissolved in a mixture of ethanol and water (1:2), then placed in a separating funnel for fractionation. The extract was then added to ethyl acetate solvent at a 1:1 ratio and left to stand until two phases formed. The ethyl acetate phase was taken to obtain the semi-polar fraction (Najmah et al., 2023).

Phytochemical Screening

Avocado leaf extracts were subjected to phytochemical screening with specific reagents to identify the presence or absence of secondary metabolite content. Dragendorff, Wagner, and Mayer reagents were used to identify alkaloid content, $FeCl_3$ for tannin, and water for the saponin test. Moreover, the flavonoid contents were examined using Mg powder and HCl. Liebermann-Buchard was used to assess steroid content in the sample (Arasaretnam et al., 2021).

Lipinski's rule of five and ADMET

It is important to study Lipinski's Rule of Five before proceeding with molecular docking calculations as an initial selection. This study aimed to determine which compounds from the avocado leaf medicinal plant comply or do not comply with Lipinski's rules. It concerns the physicochemical properties of drugs that resemble drugs (druglikeness). Lipinski's rules are drug molecular mass ≤500 Da, octanol/water partition coefficient (MlogP) ≤5, Hydrogen Bond Acceptor (HBA) ≤10, and Hydrogen Bond Donor (HBD) ≤5. Apart from that, ADMET predictions are also carried out to determine the pharmacokinetic properties of drug candidates derived from medicinal plants. The Lipinski and ADMET tests are carried out by inputting compounds into a software website-based application: http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp and https://preadmet.webservice.bmdrc.org/ (Aloui et al., 2024).

Molecular Docking Calculation

Molecular docking calculations were performed to predict ligand-COX2 complexes using a computer equipped with an Intel® Celeron N4020 CPU, 8.00 GB of installed RAM, and a 64-bit Windows 11 Home Single Language operating system. The docking process involved several vital steps. First, potential ligands derived from avocado leaves were identified and retrieved from the KNApSAcK Family database (http://www.knapsackfamily.com/), with the selected compounds listed in Table 2 (Shinbo et al., 2006). To ensure accurate structural information, three-dimensional models of these ligands were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The receptor's crystal structure of cyclooxygenase-2 (COX-2) was acquired from the Protein Data Bank (https://www.rcsb.org/) using PDB ID 5IKR. Validation of the molecular docking calculation method was performed by redocking between the native ligand and the COX-2 receptor. The best conformation should have an RMSD value of less than 2 Å. The docking calculations were executed using AutoDockTools 1.5.6 software, with the binding site defined as a cubic box of dimensions 40 Å × 40 Å × 40 Å was centered at the coordinates X=38.042, Y=2.131; and Z=61.28. The resulting docking poses were ranked based on their predicted binding affinities (kcal/mol), and the top-scoring pose for each ligand was selected for further analysis. Ligand-receptor interactions were examined using Biovia Discovery Studio. This comprehensive molecular docking approach aimed to elucidate potential interactions between avocado leaf compounds and COX-2, providing insights into their possible anti-inflammatory mechanisms (Antari et al., 2024; Utami et al., 2020).

Density Functional Theory (DFT) Calculations

DFT calculations were performed using ORCA and Avogadro software was used in the process (Kiven et al., 2024). The first molecular orbital (FMO) includes the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO), which were visualized and their energies generated using the IboView program.

Molecular Dynamics Simulation

The two compounds with the lowest energy from molecular docking will be selected for molecular dynamics simulation. Molecular dynamics simulation was performed using the YASARA Dynamic program developed by Biosciences GmbH (Land, Henrik, 2017). The simulation was run using md_runfast for 100 ns with the AMBER14 force field (Case, D. A., 2016), at a temperature of 310K to observe the stability and flexibility of the complex. The root mean square deviation (RMSD) Cα, total HBond, and radius of gyration parameters were analyzed using md_Analyze. Meanwhile, RMSF and MMPBSA energy were obtained by running md_Analyzres and md_bindenergy.

Result and Discussion

Phytochemical screening of secondary metabolites in Avocado leaves (*Persea americana* Mill.)

Phytochemical screening of avocado leaf extract (*Persea americana* Mill.) is crucial for identifying the secondary metabolites it contains, thereby providing valuable information on the plant's potential as a natural medicinal ingredient. This study used both ethanol extract and the ethyl acetate fraction for screening, adhering to the principle of 'like dissolves like' in solvent selection. Table 1 reveals that the ethanol extract dan fraksi etil asetat of avocado leaves contain alkaloids, as evidenced by positive results with three reagents: Dragendorff, Wagner, and Mayer, which produced reddish-brown, brown, and white precipitates, respectively. The white Mayer precipitate is formed due to the reaction between alkaloid nitrogen and potassium tetraiodomercurate (Silla et al., 2021). Saponin testing yielded different results for the two extracts. The ethanol extract gave positive results with the formation of stable foam 1 cm high for 3 minutes, while the ethyl acetate fraction showed negative results. This difference may be due to ethyl acetate being non-polar, which limits its extraction ability. These findings are consistent with research by Azzahra et al. (2022) which also reported no saponin detection in the ethyl acetate fraction of avocado leaves.

Table 1. Phytochemical screening using ethanol and ethyl acetate solvents of avocado leaves (*Persea americana* Mill.)

Secondary Metabolites	Reagents	Ethanol extract	Ethyl acetate fraction
Alkaloid	Drgendroff	+	+
	Mayer	+	+
	Wagner	+	+
Saponin	Aquades	+	-
Tannin	$FeCl_3$	+	+
Steroid	Lieberman burchard	+	+
Flavonoid	Mg + HCl	+	+

(+) Positive result; (-) Negative result

Table 1 also shows that the tannin test gave positive results in both ethanol and ethyl acetate, which was indicated by a blue-green color change due to the formation of a complex with Fe^{3+} (Listiana et al., 2022). The presence of steroids was confirmed by the green color of the Liebermann-Burchard reagent (Kopon et al., 2020), while the flavonoid test showed positive results detected through the formation of reddish-orange flavilium salts after the reduction of the benzopyron nucleus by Mg powder and HCl (Tuldjanah et al., 2022). This combination of properties suggests possible applications in both cellular studies and cardiovascular health. Flavonoids, classified as polyphenolic compounds, exhibit a wide range of beneficial properties. They function as powerful antioxidants (Anggun et al., 2022), show promise as anticancer agents (Bakhtra et al., 2024), and demonstrate anti-inflammatory effects. To further

elucidate the medicinal potential of these compounds, we conducted comprehensive analyses using Lipinski's rule of five, ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiling, and molecular docking tests. These advanced techniques enable a more detailed understanding of the compounds' drug-like properties, pharmacokinetics, and potential therapeutic targets, paving the way for future drug development.

Lipinski test and ADMET properties

Candidate drug compounds must meet Lipinski's rule of five, namely having a molecular weight of <500 DA, log P < 5, HBA value < 10, and HBD value must not be more than 5. The results of Lipinski's analysis of avocado leaf bioactive compounds can be seen in Table 2. The results shown in Table 2 show that 10 active compounds in avocado leaves meet Lipinski's rules, namely D-arabitol, catechol, benzoic acid, tyramine, hippuric acid, isoprene, dopamine, vanillymandelica acid, 4-hydroxy-3,5-dimethoxybenzoic acid, and avocadene. The selection of these compounds was based on the results of experimental characterization from previous studies using High-Performance Liquid Chromatography (HPLC), Gas Chromatography–Mass Spectrometry (GC-MS), and Fourier Transform Infrared Spectroscopy (FTIR) (Jaja, 2007; Tcheng et al., 2022; Ahmed et al., 2018; Agunloye et al., 2025; Trujillo-mayol et al., 2021; Younis et al., 2022). Alpha-cryptoxanthin violates Lipinski's rule, namely having a molecular mass of more than 500 DA. A drug compound that has a molecular mass above 500 DA will be difficult to distribute in blood plasma so that the drug will be toxic (Maliehe et al., 2020). Eight other compounds such as Secosubamolide, Persin, 2-(heptadecyl)furan, 10',11'-Didehydro-5,8,11',12'-tetrahydro-10'-apo-beta-carotene-3,5,8- triol, 2-pentadecylfuran, Tetrahydropersin, Alpha-cryptoxanthin, and 3-Hydroxysintaxanthin violate the Lipinski log-p rule. This violation means that drug-candidate compounds will be difficult to dissolve in the lipid bilayer, so the drug will take a long time to be distributed and adsorbed (Rauf et al., 2024). Apart from carrying out a Lipinski analysis, a drug compound must also carry out an ADMET analysis. Admet analysis results can be seen in Supplementary file.

ADMET analysis aims to determine the pharmacokinetics and toxicity of drug candidates from avocado leaves that will be consumed orally. Poor pharmacokinetics and toxicity can cause failure in the discovery of new drug candidates so that they cannot be used as medicines (Karami et al., 2022). Based on the ADMET prediction results in Table 3, most compounds exhibited high Human Intestinal Absorption (HIA) values, ranging from 72.12 to 100%. This indicates that most bioactive compounds in avocado leaves have the potential for good absorption, with the likelihood of being absorbed through the gastrointestinal tract and entering systemic circulation after oral administration. Meanwhile, Caco-2 values reflect the permeability of compounds through the intestinal epithelial membrane (Dulsat et al., 2023) for example, isoprene showed a value of 23.35 nm/second, followed by avocadene at 20.56 nm/second, indicating that these compounds possess fairly good intestinal permeability.

In terms of distribution, the Blood-Brain Barrier (BBB) parameter indicates a compound's ability to cross the blood-brain barrier. Meanwhile, the Plasma Protein Binding (PPB) value indicates the extent to which the compound binds to plasma proteins. Avocadene has the highest PPB value at 100%; it tends to remain in circulation for a longer duration, but its pharmacologically active free fraction may be lower. Conversely, tyramine has a very low PPB value of 0.45%, so it has the potential for a larger free fraction, but it may also be eliminated more quickly (Chowdhury et al., 2021). Metabolism predictions suggested that catechol, dopamine, and avocadene may inhibit CYP3A4, whereas tyramine and dopamine may inhibit CYP2D6. As CYP3A4 and CYP2D6 are major drug-metabolizing enzymes, inhibition of these enzymes may increase the risk of drug–drug interactions when co-administered with substrates of the same metabolic pathways. Most compounds were predicted to be mutagenic in the Ames test, whereas avocadene was predicted to be non-mutagenic (Gao et al., 2023). Overall, avocadene has a favorable toxicity profile compared to the other compounds. These findings are based on computational predictions and require further validation through experimental pharmacokinetic and in vivo studies.

Table 2. Lipinski of five active compounds in leaves (*Persea Americana* Mill.*)*

Compounds	Pubchem ID	Lipinski's Rule of Five					
		Molecular Mass 500 (DA)	MlogP 5	Hydrogen Bond Acceptor (HBA) 10	Hydrogen Bond Donor (HBD) 5	Violation of Lipinski	Druglike
D-Arabitol	94154	152	-2.94	5	5	0	Accepted
Catechol	289	110	1.09	2	2	0	Accepted
Benzoic acid	243	122	1	2	1	0	Accepted
Tyramine	5610	137	0.89	2	3	0	Accepted
Hippuric acid	464	179	0.50	4	2	0	Accepted
Secosubamolide	16104910	340	5.1	4	1	1	Violated
Persin	5283266	380	5.7	4	1	1	Violated
Isoprene	6557	68	1.74	0	0	0	Accepted
2-(heptadecyl)furan	183142	306	5.9	1	0	1	Violated
10',11'-Didehydro-5,8,11',12'-tetrahydro-10'-apo-beta-carotene-3,5,8-triol	163184151	412	5.73	3	3	1	Violated
2-pentadecylfuran	181250	278	6.9	1	0	1	Violated
Tetrahydropersin	10905112	384	6.1	4	1	1	Violated
Alpha-cryptoxanthin	70678559	552	11.43	1	1	2	Violated
Dopamine	681	153	0,5	3	4	0	Accepted
3-Hydroxysintaxanthin	131751826	446	8	2	1	1	Violated
Vanillylmandelic acid	1245	198	0.5	5	3	0	Accepted
"4-Hydroxy-3,5-dimethoxybenzoic acid"	10742	198	1.1	5	2	0	Accepted
Avocadene	158573	286	3.5	3	3	0	Accepted

Protein-Ligand Interaction Network Analysis

Protein-ligand interaction network analysis using the STITCH database was performed to map the relationship between bioactive compounds in avocado leaves and inflammatory pathways in Homo sapiens. The results in Figure 1 showed that phenolic compounds such as catechol and vanillylmandelic acid interact with Catechol-O-methyltransferase (COMT), which plays a role in cellular detoxification and modulation of oxidative stress. Meanwhile, benzoic acid and hippuric acid are associated with metabolic pathways that are crucial for reducing the accumulation of pro-inflammatory molecules. As a positive control, mefenamic acid directly interacted with COX-2 (PTGS2), confirming COX-2 as a primary anti-inflammatory target. However, several avocado compounds were not detected in the STITCH database due to limitations in the experimental data. However, previous studies have demonstrated that bioactive compounds in avocado can modulate inflammatory pathways by suppressing NF-κB activation, downregulating COX-2 expression, and reducing prostaglandin synthesis (Atef et al., 2023; Rosa et al., 2025). Therefore, molecular docking and molecular dynamics approaches are needed to predict the affinity and interactions of avocado compounds at the COX-2 active site at the atomic level to complement the limitations of protein network analysis (Ali et al., 2023).

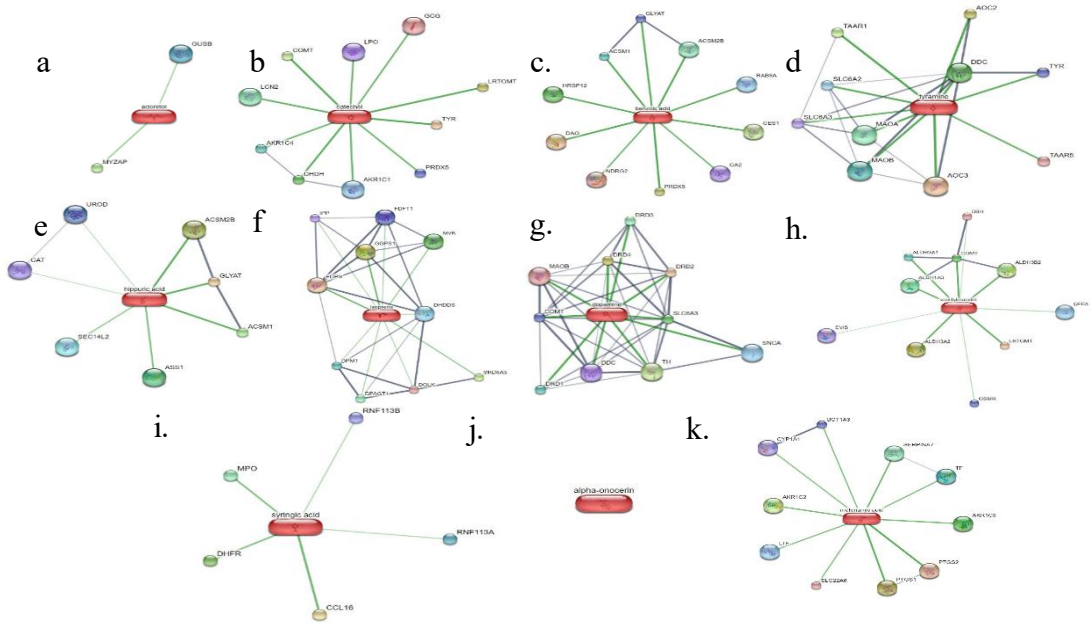


Figure 1. Interactions Protein Networks STITCH, (a) D-Arabitol; (b) Catechol; (c) Benzoic acid; (d) Tyramine; (e) Hippuric acid; (f) Isoprene; (g) Dopamine; (h) Vanillylmandelic acid; (i) 4-Hydroxy-3,5-dimethoxybenzoic acid; (j) Avocadene; (k) mefenamic acid.

Calculation of molecular docking of compounds from avocado leaves against the COX2 enzyme

The results of docking the active compound of avocado leaves with the 5IKR receptor use several parameters, namely, binding energy (kcal/mol) and the interaction that occurs between the bioactive compound of avocado leaves and the 5IKR receptor. Molecular docking can predict the microscopic interaction molecular between ligand and receptor. These interactions include hydrogen interactions, van der Waals interactions, and other interactions. The results of binding bioactive compounds in avocado leaves can be seen in Table 4. Based on Table 4, it can be seen that the binding energy of the native ligand was -8.00 kcal/mol by interaction with the amino acid residues TYR385 and SER530, which were obtained from the results of previous research (Utami et al., 2020).

Table 4. Results of docking of avocado leaf compounds with the 5IKR receptor

Compound	Binding Energy (kcal/mol)	Amino Acid Residue
Hippuric Acid	-5.4	TYR353, HIS354, ALA347, and ALA346
Catechol	-4.95	TYR353
D-Arabitol	-3.58	ASN343,PHE497,SER498
Benzoic Acid	-4.63	TYR353,SER498
Tyramin	-5.3	TYR353,THR174,ALA167
Isoprene	-3.70	-
Dopamine	-5.38	TYR353,THR174,ALA167
Vanillylmandelic Acid	-4.63	TYR353,MET490
4-Hydroxy-3,5-Dimethoxybenzoic Acid	-5.10	TYR353,MET490
Avocadene	-5.73	MET490
Asam Mefenamat (native ligand)	-8.00	TYR385, SER530

Table 4 shows that the avocado leaf compound with the lowest binding energy is the avocadane compound with a binding energy value of -5.73 kcal/mol with an amino acid residue in MET490. The binding energy between the ligand and the receptor, which shows -5 kcal/mol, indicates that the compound has potential as a disease inhibitor (Rasyid et al., 2023). A low binding energy value indicates that the bond strength between the bioactive compounds of avocado leaves and the 5IKR receptor is getting stronger. This potential prediction is supported by previous research, which states that this compound also has anti-cancer potential (Athaydes et al., 2022), anti-inflammatory, anti-thrombotic, and antioxidant (Moysidou et al., 2024). Hydrogen bonds show the most robust interactions. In the complex of avocadene compounds and COX2 receptors (Fig. 2.A.), there are two types of hydrogen bonds: intramolecular and intermolecular. Intramolecular hydrogen bonds occur between hydrogen and oxygen atoms in the avocadene compound. Meanwhile, the desired hydrogen bond is an intermolecular bond between the oxygen group from the amino acid residue MET490 and the hydrogen from the avocadene compound with a distance of 2.05 Å (Figure 2. C).

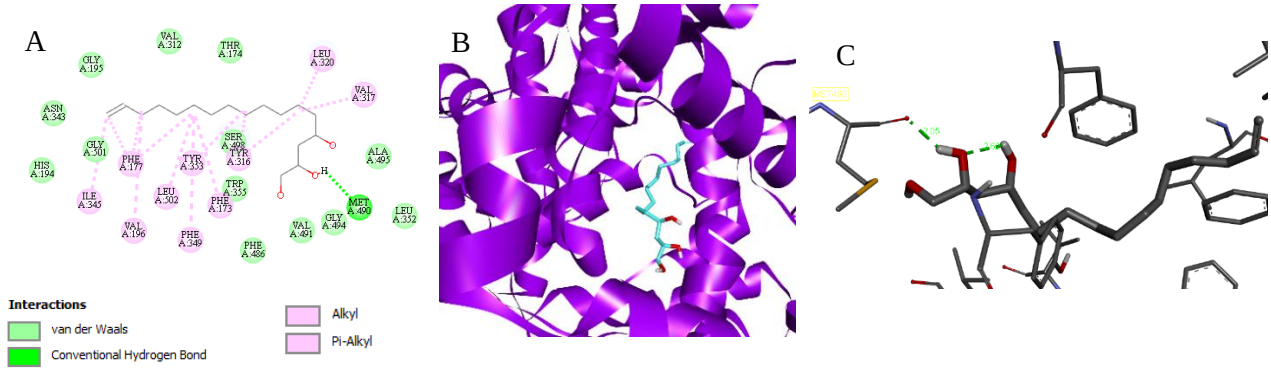


Figure 3. 2D visualization of avocadene -COX2 receptors (A), 3D visualization of the avocadene compound in the active site cavity of the COX2 receptor (B), and avocadene hydrogen bond length inhibited by TYR353 (C).

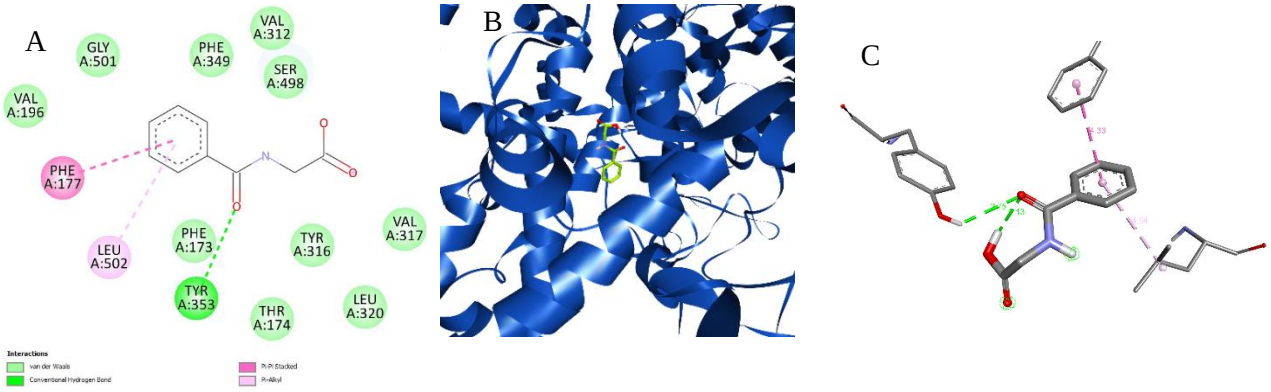


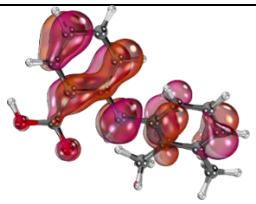
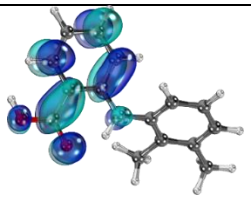
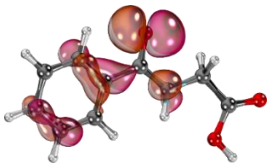
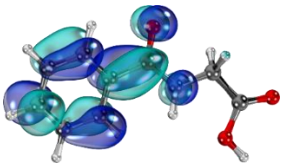
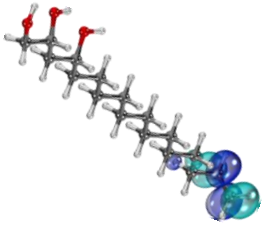
Figure 3. 2D visualization of hippuric acid-COX2 receptors (A), 3D visualization of the hippuric acid compound in the active site cavity of the COX2 receptor (B), and hippuric acid hydrogen bond length inhibited by TYR353 (C).

The next potential compound with the lowest energy is hippuric acid with BE -5.41 kcal/mol. Based on Figure 3. A, the stability of hippuric acid on the active site is enhanced by the formation of hydrogen bonds with the intervention of the amino acid residue TYR353. These hydrogen bonds comprise both intermolecular and intramolecular interactions, with bond lengths of 2.13 Å and 2.75 Å, respectively, as shown in Figure 3. C. Beyond its anti-inflammatory activity, previous studies have reported that hippuric acid also acts as a potential biomarker in reducing polycystic ovary syndrome (PCOS) pathology tested in mice (Lan et al., 2025). Apart from that, the compounds Tyramin, Dopamine, and 4-Hydroxy-3,5-Dimethoxybenzoic Acid also have binding energies close to the binding energy of avocadene and hippuric acid, namely -5.3, -5.38, and -5.10 kcal/mol, respectively.

Density Functional Theory (DFT) Calculations of Hippuric acid and Avocadene Compounds

The DFT study was conducted to evaluate the leading HOMO (Highest Occupied Molecular Orbital), LUMO (Lowest Unoccupied Molecular Orbital), and energy gap. The native ligands, avocadene and hippuric acid, have HOMO values of -6.411 eV, -6.434 eV, and -6.760 eV, respectively. The LUMO value of hippuric acid (-1.046 eV) is lower than that of avocadene (0.712 eV), indicating that it is more likely to accept electrons and therefore acts as a better electrophile (Dhande et al., 2023; Thamotharan et al., 2025). The energy difference between HOMO and LUMO is an important indicator of molecular stability and reactivity. The native ligand exhibits the lowest energy gap of 3.978 eV, consistent with its strongest docking affinity for COX-2 (-8.00 kcal/mol), whilst hippuric acid and avocadene exhibit higher energy gaps of 5.714 eV and 7.146 eV, respectively. A smaller energy gap generally indicates higher molecular reactivity, however, the affinity between the protein and the ligand is also influenced by hydrogen bonding, hydrophobic interactions, and ligand fit within the COX-2 binding site. Therefore, DFT analysis complements the docking results by identifying the electronic properties of these compounds. Visualization of HOMO, LUMO, and energy gap values is presented in Table 5.

Table 5. Interpretation of the leading orbitals of Hippuric acid and Avocadene compounds based on DFT/B3LYP calculations.

Coumpounds	HOMO (eV)	LUMO (eV)	ΔE (eV)
Mefenamic Acid (native ligand)	 -6.411	 -1.433	3.978
Hippuric Acid	 -6.760	 -1.046	5.714
Avocadene	 -6.434	 0.712	7.146

Molecular Dynamics Simulation

Molecular dynamics (MD) simulation were performed for 100 ns to evaluate the dynamic stability of the ligand-protein complex under human physiological

conditions using root mean square deviation (RMSD) Cα, radius of gyration, root mean square fluctuation (RMSF), and molecular mechanics Poisson-Boltzmann surface area **(**MMPBSA) parameters (Salo-ahen et al., 2021). RMSD is a key parameter for assessing conformational changes and the structural stability of molecular complexes during molecular dynamics simulations. As shown in Figure 4. A, the native ligand exhibits the highest stability with the lowest average RMSD of 1.705 Å, indicating that the system reached equilibrium rapidly. Similarly, avocadene and hippuric acid maintain stable trajectories < 3 Å threshold with values of 1.769 Å and 1.791 Å, respectively, without significant structural fluctuations. The radius of gyration (Rg) indicates the compactness and structural stability of the protein–ligand complexes, where increased Rg values reflect geometric expansion of the protein structure (Pražnikar, 2021). Hippuric acid exhibited moderate fluctuations around 51 ns, consistent with the RMSD profile observed during the same time interval, as shown in Figure 4. B. These results demonstrate that potential compounds maintain protein structural integrity throughout the simulation.

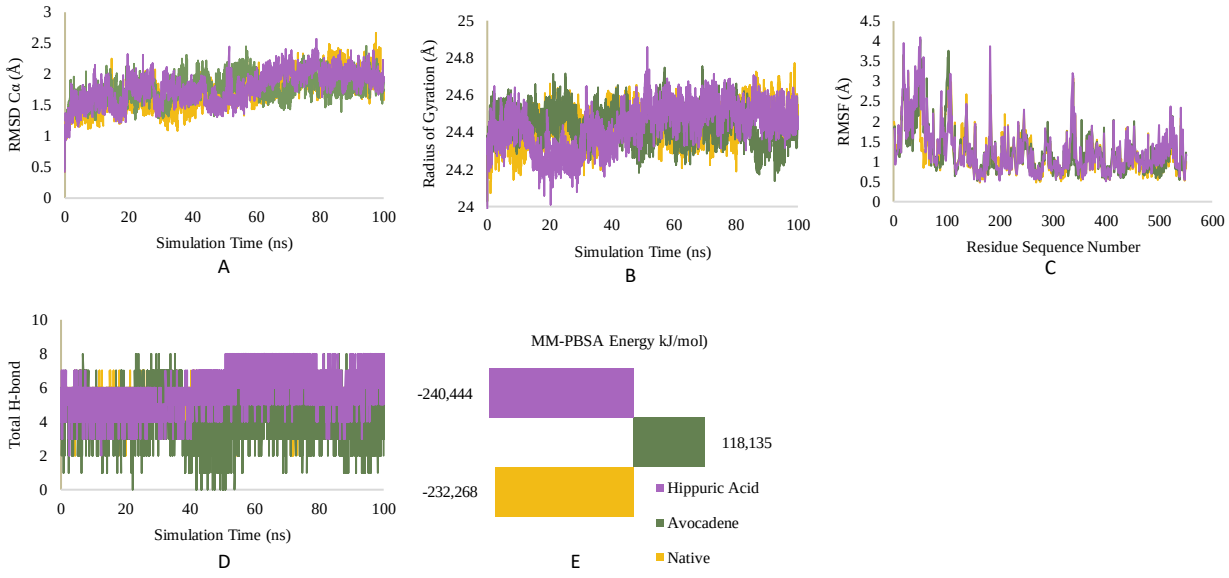


Figure 4. RMSD Cα (A), *Radius of Gyration* (B), RMSF (C), total HBond (D), and MMPBSA (E) of native ligand, avocadene, and hippuric acid.

RMSF analysis was used to evaluate the local flexibility of amino acid residues, revealing significant fluctuations in the protein backbone, particularly in the N-terminal region based on Figure 4 C (Rai et al. 2021). Hippuric acid exhibits the greatest fluctuations in residues PHE19, LYS50, HIS182, and GLN338, but because they are located outside the COX-2 active site, this movement does not interfere with the stability of the complex. The average number of hydrogen bonds reflects the strength and persistence of ligand–protein interactions, which contribute to complex stability and binding specificity (Aisyah et al., 2025; Thapa et al., 2024).

Hippuric acid has the highest value of 5.773. Furthermore, the binding energy of avocadene, as calculated by MM-PBSA, shows a positive result with a value of 118.135 kJ/mol. This contrasts with the native ligand and hippuric acid, which show negative values of -232.268 kJ/mol and -240.444 kJ/mol, respectively. Based on the MMPBSA binding energy scoring function implemented in YASARA Dynamics, more positive values indicate a stronger binding affinity between the ligand and the receptor, whereas negative values indicate unfavorable binding (Patel et al., 2021). YASARA reports the energy released upon ligand binding using a reversed sign convention. In addition, the MM/PBSA script (md_analyzebindenergy.mcr) estimates only the potential and solvation energy contributions without explicitly including the conformational entropy term (Wang et al., 2018). The results of the MMPBSA parameter are in line with the docking calculations that avocadene is the most potent compound.

Conclusion

Phytochemical research on ethanol extracts of avocado leaves (*Persea americana* Mill.) revealed various secondary compounds, including alkaloids, saponins, tannins, steroids, and flavonoids, while the ethyl acetate fraction did not contain saponins. Analysis of protein-ligand interactions using STITCH revealed that several bioactive compounds in avocado leaves interacted with proteins involved in the inflammatory pathway, and mefenamic acid directly interacted with PTGS2. Molecular docking of ten avocado leaf compounds identified avocadene and hippuric acid as potential COX-2 inhibitors, with the lowest binding energies of −5.73 kcal/mol and −5.41 kcal/mol, respectively. Key interactions were observed between avocadene and the MET490 residue, as well as between hippuric acid and the TYR353 residue of COX-2. Furthermore, the compounds exhibited favorable physicochemical and pharmacokinetic profiles, supporting its potential for oral administration. DFT analysis showed that hippuric acid has a narrower HOMO–LUMO energy gap (5.714 eV) than avocadene. Further molecular dynamics simulations confirmed the stability of both complexes, and avocadene has an MMPBSA value of 118.135 kJ/mol. Although avocadene and hippuric acid demonstrated promising inhibitory potential against COX-2, their binding affinities were lower than that of the reference ligand, mefenamic acid, which exhibited a binding energy of -8.00 kcal/mol. Therefore, these compounds may be considered potential lead compounds for further optimization and experimental validation. This research demonstrates the power of integrating phytochemical analysis with computational methods in natural product research, potentially accelerating the drug discovery process in anti-inflammatory therapeutics. The results of this study still require further confirmation through in vitro and in vivo experimental tests to determine its anti-inflammatory effects.

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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