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Secondary Metabolite Compounds from *Alpinia monopleura* Extract and Evaluation of Anti-Inflammatory Activity based on *In Vitro* and *In Silico* Studies

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ABSTRACT

***Alpinia monopleura* is one of the endemic plants of Sulawesi, and it has an extensive distribution in the region. Research on chemical compounds and biological activities of *A. monopleura* is essential to continue as an effort to support the utilization of native plants for medicine. The extract was obtained using the maceration method. The chemical compounds in the extract were identified using Liquid Chromatography Mass Spectrometry (LCMS). Bovine Serum Albumin (BSA) and molecular docking methods were used to evaluate the anti-inflammatory activity. Ten compounds contained in the extract were successfully identified, *E-para*-coumaric acid (1), *trans*-ferulaldehyde (2), 3,5,6-trihydroxy-4',7-dimethoxyflavone (3), nevadensin (4), malvalic acid (5), ent-16α,17-hydroxy-19-kauranoic acid (6), 3′,5-dihydroxy-7,4'-dimethoxy flavone (7), saurufuran B (8), 5-hydroxy-7,8,2'-trimethoxyflavanone (9) and dehydroabietic acid (10). The anti-inflammatory activity of extracts from rhizomes and stems of *A. monopleura* were 8.62 and 10.59 mg/L, respectively. Some flavonoids (9 and 7) can bind strongly to specific residues around the COX-2 active site, such as Ser530, thereby interfering with the function of the COX-2 enzyme and reducing the production of pro-inflammatory prostaglandins. Thus, *A. monopleura* extract has the potential to inhibit inflammatory responses through molecular regulation of the COX-2 enzyme.**

1. Introduction

In tropical and subtropical areas, the largest species of the Zingiberaceae family, the genus *Alpinia*, grows and spreads to 250 species (Zhang *et al.* 2016). Many countries, such as China, Japan, India, Indonesia, and Vietnam, recognize the genus *Alpinia* as a traditional medicinal plant (Van *et al.* 2021). Various biological activities such as antioxidant (Yodha *et al.* 2023), antimicrobial (Ferdous *et al.* 2018; Cruz *et al.* 2020), antiviral (Hatanaka *et al.* 2021; Narusaka *et al.* 2021; Zubair *et al.* 2021), antihypertensive (Moura *et al.* 2005), analgesic (Ahmed *et al.* 2015), neuroprotection (Mundugaru *et al.* 2018; Hashim *et al.* 2021), antiparasitic (Sulistyowaty *et al.* 2021) and anti-inflammatory (Yu *et al.* 2020) have been successfully reported.

The activity that is widely studied and interesting to do is anti-inflammatory activity. Based on previous research, it is known that the methanol extract of *Alpinia zerumbet* is able to provide anti-inflammatory activity against Nitro Oxide (NO) inhibition (Nishidono *et al.* 2019), methanol extract of *Alpinia officinarum* rhizome (Hanmore *et al.* 2016) and *Alpinia oxyphylla* extract (Yu *et al.* 2020) are able to provide anti-inflammatory activity through inhibition of cyclooxygenase 2 (COX-2), methanol extract of *Alpinia calcarata* rhizome was able to reduce the level of NO production and proinflammatory cytokines (TNF-α, IL-1β and IL-6) in RAW 264.7 cells (Erusappan *et al.* 2022), and extract from *Alpinia galanga* significantly increased anti-inflammatory cytokine IL-10 and growth factor TGF-β (Cahyono *et al.* 2023). The amount of

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information on these activities is attractive enough to continue conducting studies from the *Alpinia* genus, especially as an anti-inflammatory.

Recent research from the *Alpinia* genus, namely the *Alpinia monopleura* species, has determined its essential oil's antioxidant and antimicrobial activities. The main ingredients of the essential oil provide pharmacological activity in *in vitro* testing (Yodha *et al.* 2023).

In the Sulawesi region, *A. monopleura* is very easy to find. This plant has a wide distribution and is abundant, so it is widely used by local communities (Rugayah *et al.* 2019). Until now, only antioxidant and antimicrobial activities have been known as a form of supporting data from this utilization (Yodha *et al.* 2023). Interestingly, the biological activity of each species will be different in each part of the plant as well as its chemical compounds, caused by differences in geographical location and climate changes that occur (Mahdavi *et al.* 2017). Thus, studies on the chemical compounds and biological activities of *A. monopleura* are essential to continue to support the utilization of native plant materials.

2. Materials and Methods

2.1. Plant Material

Alpinia monopleura rhizomes and stems were obtained from Anduna Village, Laeya District, South Konawe Regency, Southeast Sulawesi Province (4°15'17.4"S 122°29'20.6"E). The BRIN Research Center for Biology determined the sample and registered it with Number (No. 951/IPH.1.01/If.07/V/2019) (Figures 1 and 2). Samples were prepared and dried at temperatures below 45°C for 7 days. The dried simplicia was pulverized and stored in a dark and dry container.

2.2. Extraction

Dry simplicia of *A. monopleura* rhizomes and stems were each macerated with methanol (Merck) for 72 hours. The filtrate was concentrated using a vacuum rotary evaporator (Stuart RE300, USA) at 50°C at 85 rpm.

2.3. Identification of Secondary Metabolite Compounds

Secondary metabolites in each extract were analyzed using a Liquid Chromatography Mass Spectrometer (LCMS) (Waters, USA). The sample injection volume was 1 µL. The stationary phase used a reversed-phase column (HSS T3 C18) at 40°C. A mixture of 0.1% Formic Acid in water (Thermo Scientific™) was used as Mobile Phase A, and a mixture of 0.1% Formic Acid in Acetonitrile (Thermo Scientific™) as Mobile Phase B. Elution followed a gradient of 10% B (0-8 min), 50% B (8-14 min) and 100% B (14-16 min) at a rate of 0.300 ml/min. The data range was collected between 50-1,200 m/z.

2.4. Inhibition of Protein Denaturation

The anti-inflammatory activity of *A. monopleura* rhizome and stem extracts was determined using a modified BSA assay based on the Bailey-Shaw report (Bailey-Shaw *et al.* 2017). Bovine serum albumin (HIMEDIA®) solution (0.2%, b/v) was prepared in tris buffered saline (HIMEDIA®) (A total of 4.35 grams of sodium chloride (Merck) and 0.6 grams of tris base (HIMEDIA®) and then dissolved with distilled water to 450 ml. Adjust pH with glacial acetic acid (Merck) to pH 6.5 (pathological pH), then add distilled water to 500 ml). Each extract was prepared in a concentration of 100 mg/L in methanol (Merck) solvent (stock solution). Aliquots of 10 µL, 20 µL, 30 µL, 40 µL and 50 µL representing concentrations of 1 mg/L, 2 mg/L, 3 mg/L, 4 mg/L, and 5 mg/L of stock solution, respectively, were added to test tubes containing 1 mL of 0.2% BSA buffer solution. Methanol (Merck) (negative control) and sodium diclofenac (Sigma-Aldrich) (positive control) were tested similarly. The solutions were then heated at 72°C for 20 min and cooled for 30 minutes under room conditions. The turbidity was measured at 660 nm. The experiment was performed in duplicate, and the average value of the absorbance was recorded. The percentage inhibition using the following equation:

$$\text{Anti-inflammatory activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.5. Molecular Docking

The research utilizes the molecular target, which is the enzyme cyclooxygenase-2 (COX-2), with the PDB code 1PXX (Rowlinson *et al.* 2003). This enzyme was obtained from the RCSB website (https://www.rcsb.org/). After getting the target, we prepared it for further analysis using AutoDock Tools v1.5.6. This preparation involved the removal of chains B to E, the ligands, and water molecules from the enzyme structure. Additionally, we added hydrogen atoms to

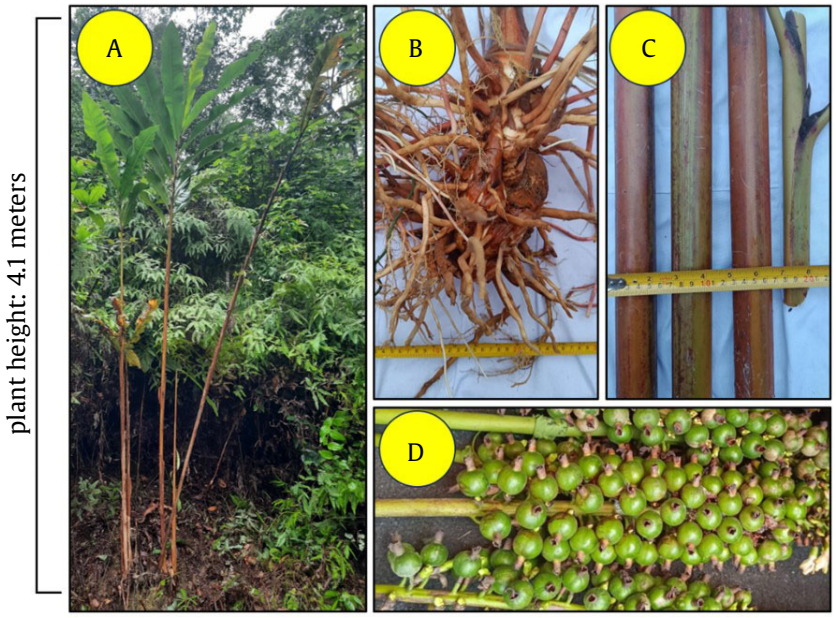

Figure 1. *Alpinia monopleura* (A) plant, (B) rhizome, (C) stem, and (D) fruit

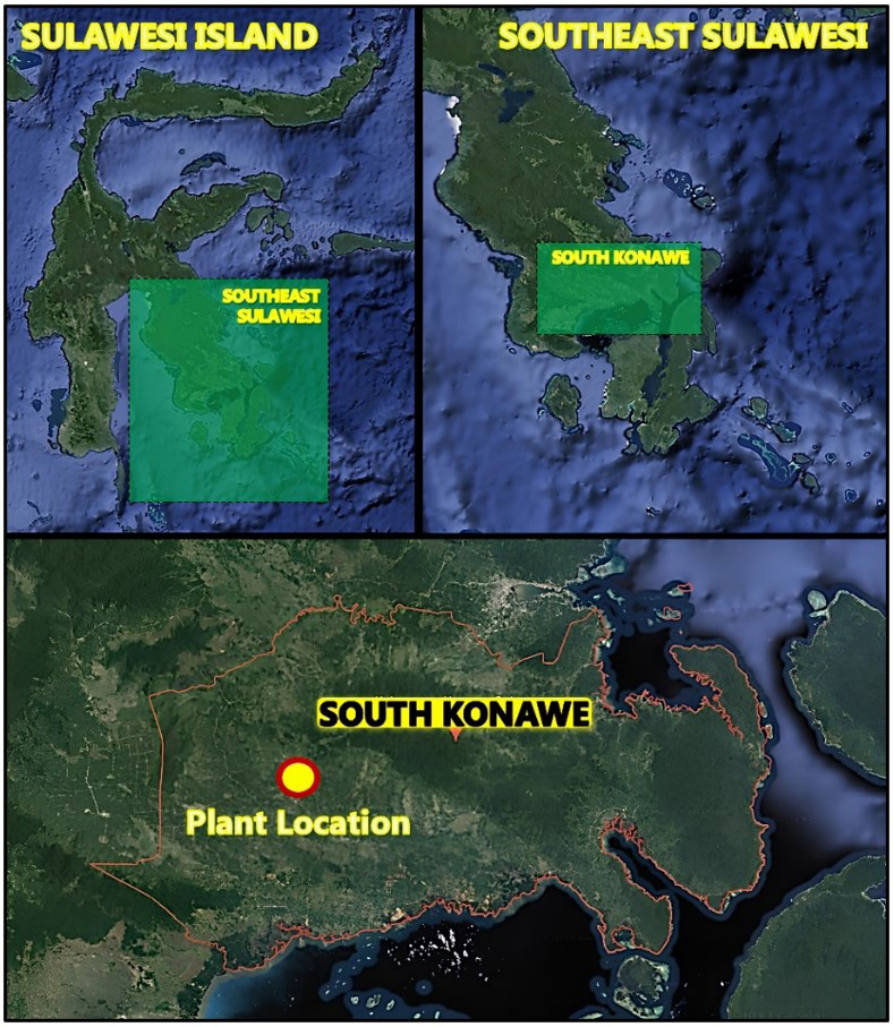

Figure 2. *Alpinia monopleura* were obtained from Anduna Village, Laeya District, South Konawe Regency, Southeast Sulawesi Province (4°15'17.4"S 122°29'20.6"E)

the polar groups of the enzyme structure and applied Kollman charges as needed for the analysis (Morris *et al.* 2009).

In the next stage, a 3D structure of the compounds was needed to study their molecular mechanism against the COX-2 enzyme. The presence of flavonoid compounds influenced the anti-inflammatory activity derived from natural products (Ribeiro *et al.* 2015). Four flavonoid compounds were identified from *A. monopleura* based on LC-MS results, namely [3,5,6-trihydroxy-4'7-dimethoxyflavone (3), nevadensin (4), 3',5-dihydroxy-7,4'-dimethoxyflavone (7), and 5-hydro-7,8,2'-trimethoxyflavanone (9)]. These compounds were used as test compounds for simulations against the COX-2 enzyme. The 3D structures of these flavonoid compounds were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The test ligands were then prepared further for analysis, which included adjustments to their maximum torsional conformation, adding hydrogen atoms, and applying Gasteiger charges using AutoDock Tools v1.5.6 (Morris *et al.* 2009).

The molecular docking procedure was carried out using AutoDock 4.2, utilizing a cubic grid measuring 40 Å with its center-coordinated position of diclofenac in the COX-2 active site. Genetic Algorithms with 100 conformational searches and a maximum of 2,500,000 evaluations were applied to find the best molecular docking parameters (Morris *et al.* 2008). Diclofenac was docked onto COX-2 to validate the initial genetic algorithms. The reliability of the initial docking method is confirmed if the redocked conformation of the diclofenac exhibits a Root Mean Square Deviation (RMSD) value of ≤ 2 Å compared to its crystallographic conformation (Arfan *et al.* 2022). The molecular docking analysis was based on selecting ligand configurations with the most favorable binding free energy (ΔG) and visualizing their interaction with COX-2 using Discovery Studio Visualizer software v17.2.0.16349.

3. Results

The extraction results showed that the extract content of the rhizomes and stems of *A. monopleura* were 1.80% and 2.35%, respectively (Table 1).

The chromatogram of the analysis of chemical compounds from each extract (Figure 3) based on LCMS data observed from Retention Time (RT) 0-16 minutes shows separate peaks. Each peak represents the compounds contained in the extract and is separated due to differences in distribution on certain mobile and stationary phases.

Table 1. *Alpinia monopleura* rhizomes and stems extract levels

Sample	Weight of simplicia (g)	Extract (g)	Yield (%)
Rhizomes	400	7.2	1.80
Stems	400	9.4	2.35

Each peak observed through Electrospray Ionization Mass Spectrometry forms a mass-to-charge ratio (m/z) value so that its molecular weight can be confirmed. The most probable molecular formula as the composition of the compound structure was deduced using databases in Metlin, Metfrag, Massbank North America, Massbank Japan, mzCloud, ChemSpider, and PubChem.

The content of chemical compounds in the extracts of rhizomes and stems of *A. monopleura* is presented in Table 2. A total of 10 chemical compounds that were successfully identified (Figure 4) were distributed in each extract with different concentrations, molecular structures, and compound groups.

The data in Figure 5 presents the results of the anti-inflammatory test using the protein denaturation method. The inhibition values of the rhizome and stem extracts of *A. monopleura* and sodium diclofenac were 8.62, 10.59, and 5.49 mg/L, respectively.

The potential of flavonoid compounds in *A. monopleura* as anti-inflammatory agents was strengthened through testing their interaction with the COX-2 enzyme. Examining these molecular interactions utilizes a validated docking simulation that successfully mimics the binding conformation of diclofenac to the active site of COX-2 based on an RMSD criterion of 1.839 Å (Figure 6).

After a comprehensive study of the docking results, binding energy analysis can aid in understanding the potential of flavonoid compounds to inhibit COX-2 enzymes. The analysis revealed that diclofenac has the lowest binding energy compared to flavonoid compounds at -7.85 kcal/mol against COX-2. The lower the binding energy, the stronger the binding between the compound and the COX-2 enzyme. This causality could imply that the compound is more effective in inhibiting COX-2 enzyme activity. Meanwhile, compounds **9**, **7**, **3**, and **4** have binding energies of -6.87 kcal/mol, -6.37 kcal/mol, -6.24 kcal/mol, and -6.07 kcal/mol, respectively (Table 3).

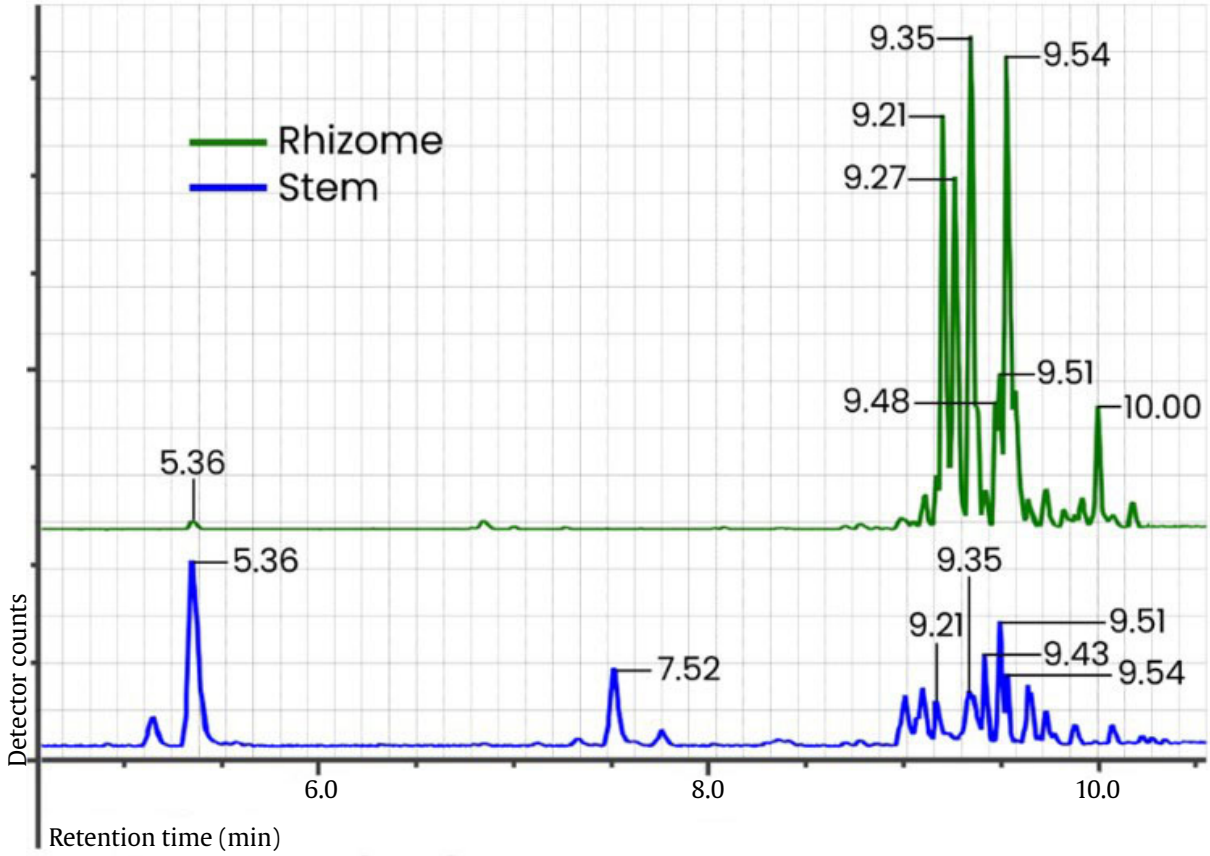

Figure 3. LC-MS chromatogram of the *A. monopleura* rhizomes and stem extract

Table 2. Identification of secondary metabolites in the *A. monopleura* rhizomes and stems extract

Observed RT (min)	Observed $[M+H]^+$ m/z	Rhizomes	Stems	Group
5.36	165.0545	*E-para*-coumaric acid	*E-para*-coumaric acid	Phenolic
7.52	179.0703	not identified	*trans*-ferulaldehyde	Phenolic
9.21	331.0818	3,5,6-trihydroxy-4',7-dimethoxyflavone	3,5,6-trihydroxy-4',7-dimethoxyflavone	Flavonoid
9.27	345.0977	nevadensin	not identified	Flavonoid
9.35	281.2478	malvalic acid	malvalic acid	Fatty acid
9.43	321.2427	not identified	ent-16α,17-hydroxy-19-kauranoic acid	Terpenoid
9.48	315.0867	3′,5-dihydroxy-7,4'-dimethoxy flavone	not identified	Flavonoid
9.51	317.2119	saurufuran B	saurufuran B	Terpenoid
9.54	329.1025	5-hydroxy-7,8,2'-trimethoxyflavanone	5-hydroxy-7,8,2'-trimethoxyflavanone	Flavonoid
10.00	301.2166	dehydroabietic acid	not identified	Terpenoid

Sodium diclofenac typically forms hydrogen bond (H-bond) interactions with Ser530 on the COX-2 active site (Figure 6), also observed in compounds **9**, **7**, and **3** (Figures 7A, B, and C). Compounds 9 and 3 also exhibit additional H-bond interactions with residues Ser533 and Tyr385. Interestingly, compound **4** shows three distinct H-bond interactions with different residues compared to the other compounds, namely Arg120, Tyr355, and Met522 (Figure 7D).

Additionally, all compounds establish hydrophobic interactions with the Val349, Val523, Ala527, and Leu531 amino acid residues at the COX-2 active site. These amino acid residues are located around the active site of the COX-2 and play a role in shaping the structure that supports interactions with substrates and the enzymatic activity of COX-2. A unique observation was found in compounds **9**, **7**, and **3**, which exhibit Pi-cation interactions with the

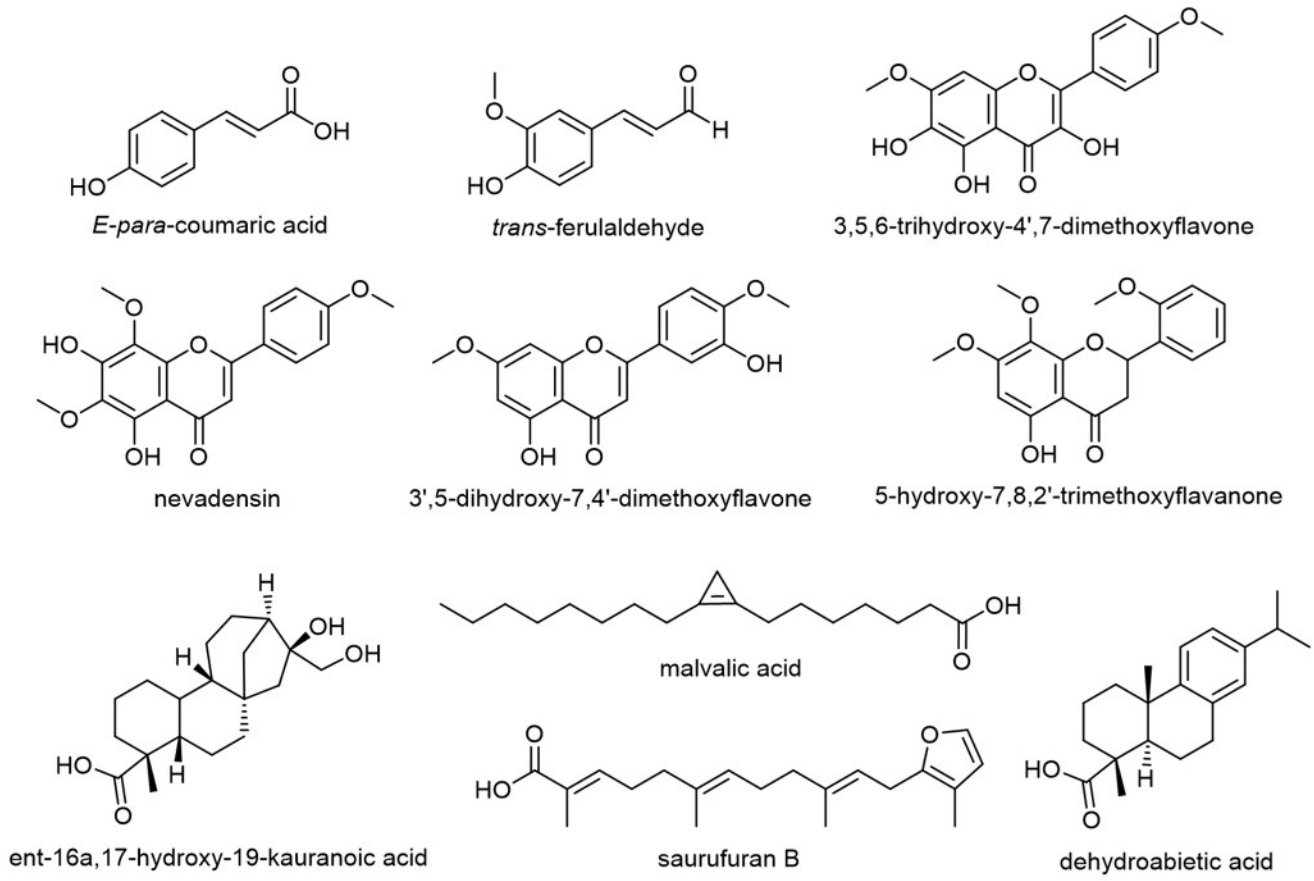

Figure 4. Structure secondary metabolites of the *A. monopleura*

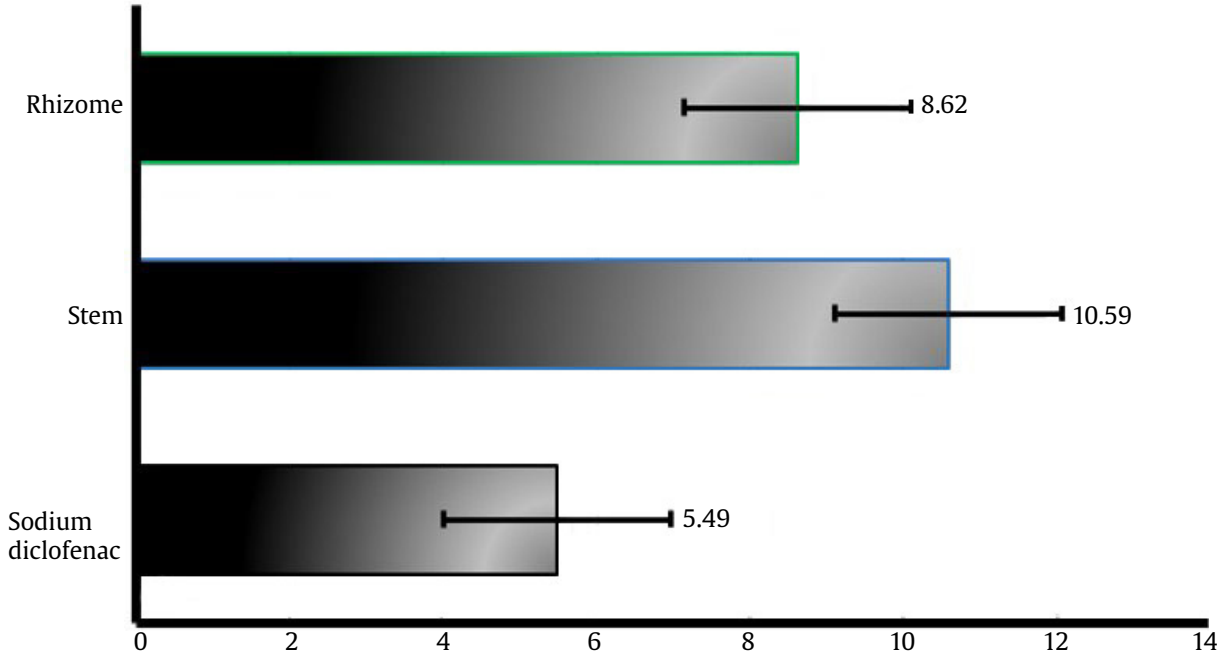

Figure 5. Determination of anti-inflammatory activity in *A. monopleura* extract

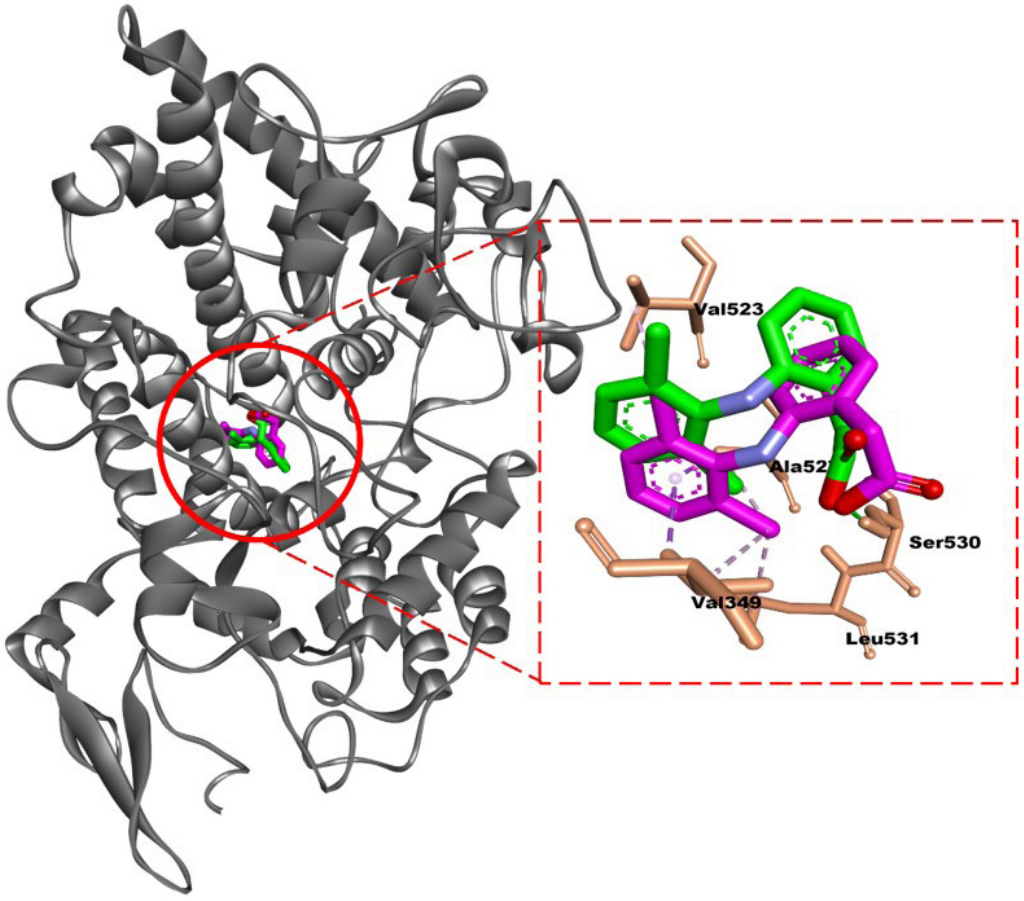

Figure 6. Overlay of the crystallographic conformation of diclofenac (pink) with the best-redocked conformation of diclofenac (green) on the COX-2 enzyme

Table 3. Summary of binding energy and interactions of the flavonoid compounds from *A. monopleara* with COX-2

Compounds	Binding energies (kcal/mol)	Hydrogen bonds	Hydrophobic interactions
Sodium Diclofenac	-7.85	Ser530	Val349, Val523, Ala527, Leu531
(9)	-6.87	Ser530, Ser533	Arg120, Val349, Gly526, Ala527, Met522
(7)	-6.37	Ser530	Arg120, Val349, Ala527, Leu531
(3)	-6.24	Ser530, Tyr385	Arg120, Val349, Ala527, Leu531
(4)	-6.07	Arg120, Tyr355, Met522	Val349, Gly526, Ala527, Leu531

Arg120 residue that were not observed in diclofenac interactions (Figure 7A, B, and C).

4. Discussion

The level of extract produced is influenced by the ability of methanol as an extraction solvent, which can bind many groups of metabolite compounds of polar and nonpolar ones, such as terpenoids, saponins, phenolics, tannins, and flavonoids (Fonmboh *et al.* 2020). Resulting levels is smaller than the results of the same genus, *A. galanga*, which showed the results of rhizome and stem extracts of 3.7% (Pradani *et al.* 2024) and 5.2% (Tangkau *et al.* 2023), respectively. The difference was caused by differences in samples (Cahyono *et al.* 2020). In addition, previous research explained that the *Alpinia* genus contains nonpolar compounds of 32-57% (Hernani *et al.* 2007), so in general the low yield value is thought to be caused by methanol as an extraction solvent which is polar so that it cannot extract all active compounds. These secondary metabolite compounds consist of two phenolics [*E-para*-coumaric acid (1), *trans*-ferulaldehyde (2)], one fatty acid [malvalic acid (5)], three terpenoids [ent-16α,17-hydroxy-19-kauranoic

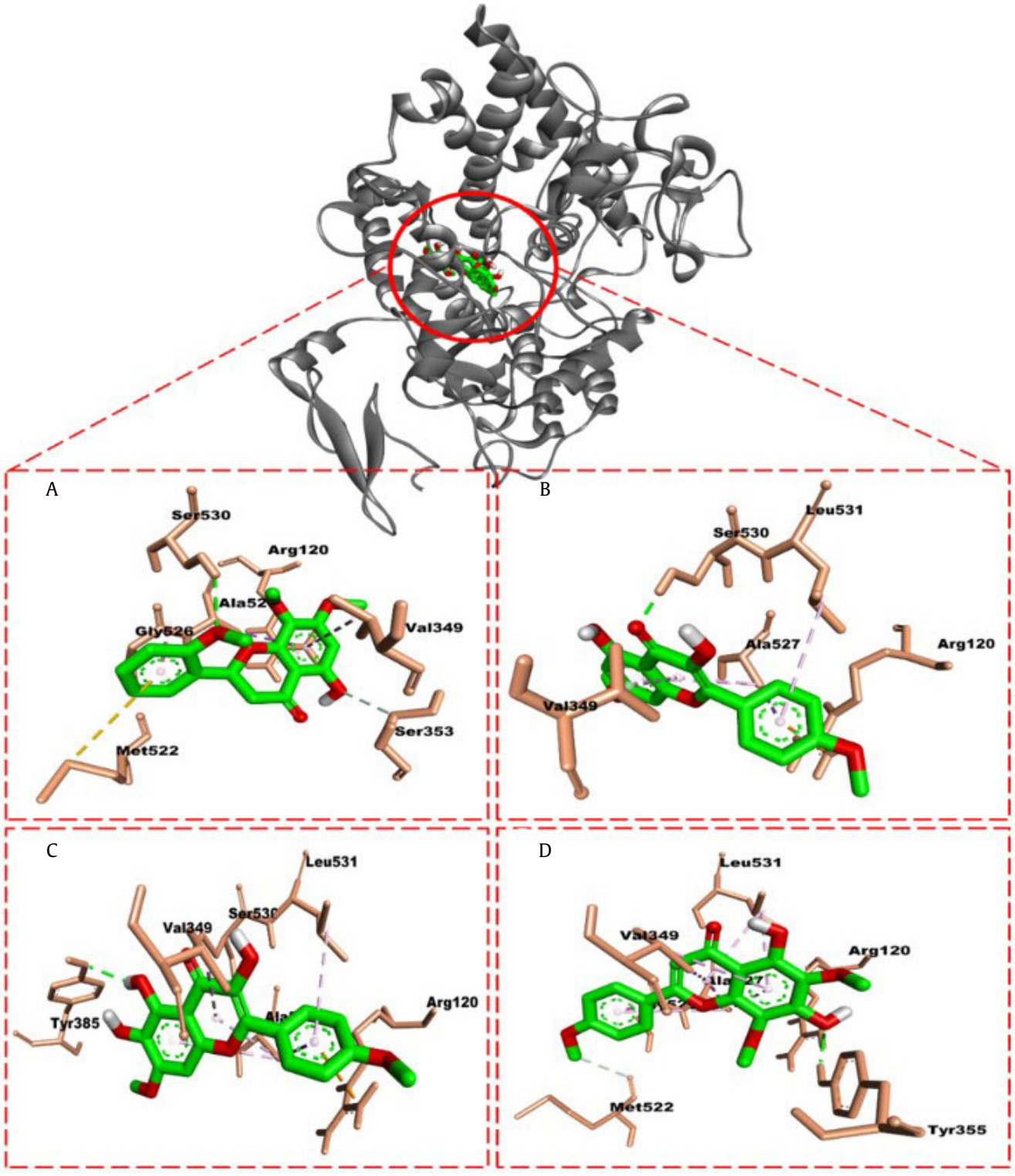

Figure 7. Molecular interactions of (A) compound 9, (B) compound 7, (C) compound 3, and (D) compound 4 on the COX-2 active site

acid (6), saurufuran B (8), dehydroabietic acid (10)] and four flavonoids [3,5,6-trihydroxy-4',7-dimethoxyflavone (3), nevadensin (4), 3',5-dihydroxy-7,4'-dimethoxy flavone (7), 5-hydroxy-7,8,2'-trimethoxyflavanone (9)].

Testing the antiinflammation activity of the extracts was carried out with the protein denaturation inhibition method using Bovin Serum Albumin. This method is used because protein denaturation in tissues is one of the causes of inflammation. Heat can be used to affect hydrogen bonds and non-polar hydrophobic interactions because heat increases kinetic energy and causes the molecules that make up the protein to move so fast that it disrupts hydrogen bonds. In addition, heating will change the protein's water-binding ability. Heat energy will result in the breaking of non-covalent interactions that exist in the natural structure of the protein but do not break its covalent bonds in the form of peptide bonds. This process occurs over a narrow temperature range. Inhibition of protein denaturation is known by measuring the absorption by UV-Vis spectrophotometry. Extracts that inhibit protein denaturation greater than 20% are considered to have anti-inflammatory activity and can be used as reference values for drug development (Verma *et al.* 2011). Specifically, an extract is declared as a very strong anti-inflammatory if IC_{50} is less than 10 mg/L, strong if IC_{50} is 10-30 mg/L, moderate if IC_{50} is 31-50 mg/L, weak if IC_{50} is 51-100 mg/L and inactive if IC_{50} is more than 100 mg/L. Rhizome extracts have better activity than stem extracts, but based on IC_{50} values, *A. monopleura* rhizome and stem extracts have anti-inflammatory activity with a very strong category (Musdalipah *et al.* 2023).

Flavonoid compounds become compounds with the most groups in the extract to dominate the chemical content. The dominance of the content is closely related to the biological activity produced from each extract. The content of flavonoids is thought to influence the anti-inflammatory activity of the tested extracts. This explains the better ability of the rhizome extract than the stem extract. Flavonoids are known to have good anti-inflammatory activity in terms of their molecular structure. The planar ring structure and the position of the hydroxyl group are vital for providing such activity (Shamsudin *et al.* 2022). Flavonoid compounds exhibit significant potential as anti-inflammatory agents due to their capability to inhibit pro-inflammatory enzymes, such as cyclooxygenase (COX-2) and lipoxygenase (LOX). These enzymes are pivotal in generating inflammatory mediators (Al-Khayri *et al.* 2022).

We conducted a molecular docking simulation study on the content of flavonoid compounds from the *A. monopleura* plant. Some noteworthy flavonoid from *A. monopleura*, called compounds 9 and 7, demonstrates a strong affinity towards specific residues on the COX-2 active site, exhibiting a binding energy of -6.87 kcal/mol and -6.37 kcal/mol, respectively. Binding energy describes how strongly a compound or molecule can bind to the enzyme (Citra *et al.* 2023). The interactions between compounds **9** and **7** with COX-2 are supported by the involvement of carbonyl groups within the compound, which engage with the amino acid residue Ser530. Ser530 is a crucial constituent of the COX-2 active site and catalyzes the transfer of hydroxyl groups to arachidonic acid during the initial stages of prostaglandin synthesis (Rouzer and Marnett 2020. Furthermore, compounds 9 and 7 exhibit hydrophobic interactions with another essential residue, Ala527. This residue plays a pivotal role in molecular interactions with the COX-2 enzyme. Some of them, such as Ala527, can influence the catalytic activity of COX-2 through interactions with other residues, like Ser530, which is crucial in COX-2 catalysis (Rowlinson *et al.* 2003). These intricate interactions collectively disrupt the normal functioning of the COX-2 enzyme, thereby diminishing its capacity to generate pro-inflammatory prostaglandins. Based on these findings, flavonoid compounds derived from this plant show the potential to effectively reduce inflammatory responses by modulating the catalytic activity of the COX-2 enzyme.

In conclusion, the study successfully identified ten compounds within the *A. monopleura* extract, revealing that extracts from the rhizomes and stems of *A. monopleura* exhibit prominent anti-inflammatory properties. Two flavonoid compounds from this plant, compounds 9 and 7, showed strong interactions with COX-2, indicating their potential to reduce inflammation effectively. These findings open up vast opportunities to explore natural plant compounds as potential sources of therapeutic agents to address inflammation.

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