Berkala **PENELITIAN HAYATI**

ISSN: 08526834 | E-ISSN:2337-389X

Volume 32 | No. 1 | **April** | 2026

Original article

In Silico and In Vivo Analysis of the Therapeutic Potential of *Zingiber americanus* Water Extract

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Abstract

Zingiber americanus (lempuyang emprit) is a medicinal plant from the Zingiberaceae family that contains flavonoids, phenolics, terpenoids, and essential oils. Traditionally, it has been used to treat pain, inflammation, and digestive disorders. This study evaluated the analgesic activity of *Zingiber americanus* water extract using in silico and in vivo methods. In the in silico study, bioactive compounds identified by LC-HRMS were tested against an inflammation-related protein (PDB ID: 1RWK) using AutoDock Vina, while pharmacokinetic properties were analyzed using SwissADME. In the in vivo study, male rats were treated with extract doses of 100, 200, and 300 mg/kg BW. Analgesic activity was measured using the writhing reflex method induced by 1% acetic acid. The results showed that alpinetin and (+)-pinocembrin had the strongest binding affinity (−4.6 kcal/mol), better than the native ligand (−3.9 kcal/mol). The extract also reduced writhing responses in a dose-dependent manner, with the 300 mg/kg BW dose showing the strongest analgesic effect, although still weaker than meloxicam. These findings indicate that *Zingiber americanus* has promising analgesic potential supported by computational and experimental studies.

Keywords: analgesic, anti-inflammatory, caspase-1, molecular docking, writhing reflex, *Zingiber americanus*

Received: December 17, 2025 | Revised: April 4, 2026 | Accepted: April 30, 2026

Introduction

The World Health Organization estimates that 80% of the Indonesian population still relies on traditional medicine, using traditional alternative medicines. Indonesians prefer herbal medicines because they have no side effects or cause relatively minor side effects (Andini DAP, et al., 2024). The use of traditional medicine is supported by abundant natural resources, making it easier for people to use traditional medicines, especially in rural areas (Budiyanti LE, et al., 2024). The sensation of pain can be triggered by mechanical, chemical, or physical stimuli that cause tissue damage. These stimuli trigger the release of pain mediators such as bradykinin, histamine, leukotrienes, and prostaglandins. Pain can result in certain responses if not managed and allowed to persist. The responses that may occur include increased anxiety, respiratory rate, blood pressure, heart rate, and can reduce immune function, thereby potentially causing tissue damage (Hidayat, T.S., et al., 2024).

Research on *Zingiber americanus* (lempuyang emprit) is important due to its potential as a source of bioactive compounds with anti-inflammatory and analgesic activities, similar to other members of the Zingiberaceae family. Previous studies reported that compounds such as zerumbone, gingerol, and shogaol can inhibit inflammatory mediators, including NO, NF-κB, COX-2, and pro-inflammatory cytokines (Chien et al., 2008; Yuandani et al., 2023). Recent findings also showed that bioactive compounds from Zingiber species regulate inflammasome-related pathways and immune responses, supporting their potential as natural anti-inflammatory agents (Pázmándi et al., 2024). However, studies on the molecular mechanism of *Zingiber americanus* are still limited. To the best of our knowledge, no previous study has specifically investigated the interaction of its bioactive compounds with caspase-1, a key regulator in inflammatory pain signaling. Therefore, this study combines molecular docking targeting caspase-1 and in vivo analgesic evaluation to investigate the pharmacological potential *of Zingiber americanus.* The identification of compounds with favorable binding affinity toward caspase-1 is expected to provide new insights into its analgesic and anti-inflammatory mechanisms.

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Methods

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals and approved by the Ethics Committee of Islamic Hospital [Rumah Sakit Islam UNISMA] (No. 80/KEPK/RSI-U/XI/2025).

Extraction

The lempuyang emprit powder was weighed at 100 grams and dissolved in 1500 mililiters of distilled water and extracted using UAE for 20 minutes. The extract was

http://dx.doi.org/10.23869/bphjbr.32.1.20267
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then filtered. The extract was freeze-dried to yield a sticky and brown extract (Maharani et al., 2022).

Phytochemicals Screening

The extract was tested for possible constitution of various natural products, such as flavonoids, phenols, alkaloids, tanins, terpenes, and saponins. Identification of compound content is carried out using LC-HRMS.

Drug Likeness Analysis

In silico ADME screening and drug likeness evaluation were conducted using the free web tool SwissADME, developed by the Swiss Institute of Bioinformatics and freely available at www.swissadme.ch. Simple physicochemical profiles such as molecular weight (MW), molecular refractivity (MR), number of atoms, and polar surface area (PSA) were calculated. Drug-likeness was assessed using the criteria of Lipinski, Ghose, Veber, Egan, and Muegge. Compounds that meet all five of these criteria will undergo docking.

Molecular Docking Active Compound

The 3D structure of the target protein (PDB ID: 1RWK) was downloaded from the RCSB Protein Data Bank in .pdb format. Protein preparation was carried out using BIOVIA Discovery Studio by removing water molecules and native ligands, separating chain A, and adding polar hydrogen atoms. Chain A was used as the receptor in this study.

From 26 compounds identified in *Zingiber americanus* Rhizome, six compounds met the drug-likeness and ADMET criteria and were selected as ligands. Their 3D structures were obtained from PubChem in .sdf format and optimized using PyRx before docking.

Molecular docking was performed using PyRx by inserting the prepared protein and ligands. The binding site was adjusted to the active site of the protein. The docking results were evaluated based on binding affinity values, where lower energy indicated stronger interactions. The docking complexes were visualized using BIOVIA Discovery Studio to observe hydrogen bonds, hydrophobic interactions, and electrostatic interactions between the ligands and the protein active site.

Writhing Test

First, all test animals were fasted for 6-8 hours. Emptying the stomach is beneficial for the drug absorption process. The presence of food in the stomach often interferes with absorption, thereby affecting drug efficacy. Then, the rats' body weights were measured and recorded. The rats were divided into 5 treatment groups, namely the negative control group, positive control group, and treatment groups with doses 1, 2, and 3. The positive control group was given meloxicam suspension at a dose of 50 mg/kg body weight, whereas the test groups were given lempuyang extract suspension at 100 mg/kg, 200 mg/kg, and 300 mg/kg body weight. After administering the test substances by gavage, a 30-minute waiting period was allowed. Then, glacial acetic acid was induced intraperitoneally. After 5 minutes, the rats experienced pain, showing writhing effects. The number of writhes was observed and recorded every 5 minutes for 60 minutes. A graph of writhing response versus time was then plotted.

$$\% \text{ inhibition} = \frac{(\text{Total writhes in the control group} - \text{total writhes in the test group})}{\text{Total writhes in the control group}} \times 100\%$$

Statistical Analysis

The analysis was conducted using statistical package for social science (SPSS). One way ANOVA was used to analyze the data followed by Tukey's post hoc test to determine statistical significance. All the data were expressed as mean +/- standard error of the mean (SEM). A P-value <0.05 was taken as statistically significant.

Results

Phytochemical Screening

The phytochemical analysis exhibited that water extract of lempuyang emprit Rhizome contains flavonoids, phenols, and tannins. The compounds from lempuyang emprit Rhizomes were identified using LC-HRMS, and their identities are listed in Table 1. Among the identified compounds, only six met the defined drug-likeness and ADMET criteria and were selected for molecular docking analysis.

Table 1. The Compounds of Lempuyang Emprit Rhizome

Active compounds	CID	SMILES structure
Dl-Tryptophan	1148	C1=CC=C2C(=C1)C(=CN2)CC(C(=O)O)N
Alpinetin	4053302	COC1=CC(=CC2=C1C(=O)CC(O2)C3=CC=CC=C3)O
(+/-)-Pinostrobin	4101463	COC1=CC(=C2C(=O)CC(OC2=C1)C3=CC=CC=C3)O
Kaempferol	5280863	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O
2',4,4',6'-Tetrahydroxychalcone	5280960	C1=CC(=CC=C1/C=C/C(=O)C2=C(C=C(C=C2O)O)O)O
(+)-Pinocembrin	68071	C1[C@H](OC2=CC(=CC(=C2C1=O)O)O)C3=CC=CC=C3

Table 2 shows that most active compounds meet the druglikeness criteria based on the Lipinski, Ghose, Veber, Egan, and Muegge rules, indicating a high potential as oral drug candidate. Flavonoid compounds such as alpinetin, pinostrobin, kaempferol, and tetrahydroxychalcone meet all the criteria, demonstrating good bioavailability and stability. In contrast, compounds such as afzelin, adenine, and vanillin do not meet some rules due to high polarity or molecular weight. Overall, flavonoid and terpenoid compounds from Zingiberaceae exhibit the best pharmacokinetic profiles and have potential to be developed as natural anti-inflammatory and immunomodulatory agents.

Table 2. Drug Likeness Analysis

Active compounds	Druglikeness				
	Lipinski	Ghose	Veber	Egan	Muegge
Dl-Tryptophan	Yes	Yes	Yes	Yes	Yes
Alpinetin	Yes	Yes	Yes	Yes	Yes
(+/-)-Pinostrobin	Yes	Yes	Yes	Yes	Yes
Kaempferol	Yes	Yes	Yes	Yes	Yes
2',4,4',6NoTetrahydroxychalcone	Yes	Yes	Yes	Yes	Yes
(+)-Pinocembrin	Yes	Yes	Yes	Yes	Yes

The Table 3 shows the results of molecular docking between several active compounds and the target receptor. Lower binding energy values indicate stronger affinity. Alpinetin and (+)-pinocembrin have the highest affinity (−4.6 kcal/mol), followed by DL-tryptophan (−4.4 kcal/mol). All compounds interact with key residues such as HIS237, ARG179, and CYS285, which are also involved with the natural ligand. These results suggest that these compounds have potential biological activity, particularly as anti-inflammatory agents.

Table 3. The Lempuyang Emprit Rhizome compound interaction with ICE (1RWK)

Active compounds	Est. Free Energy of Binding (Kcal/mol)	Amino Acid Residue
Native Ligand	-3.9	GLN283 CYS285 HIS237 ARG179
Dl-Tryptophan	-4.4	SER236 GLN283 ARG179 HIS237 CYS285
Alpinetin	-4.6	HIS237 ARG179 CYS285 GLN283
(+/-)-Pinostrobin	-3.8	HIS237 ARG179 SER236 CYS285
Kaempferol	-3.8	SER236 HIS237 ARG179 CYS285
2',4,4',6NoTetrahydroxy-chalcone	-3.4	CYS285
(+)-Pinocembrin	-4.6	HIS237 GLN283 SER236 ARG179 CYS285

Writhing Test

The acetic acid–induced writhing test demonstrated that the water extract of lempuyang emprit Rhizome produced a dose-dependent reduction in the number of writhes compared with the negative control group. The highest dose (300 mg/kg) showed the greatest analgesic effect, with 98.86% inhibition ($p < 0.05$), comparable to the positive control meloxicam (98.62% inhibition). The 200 mg/kg dose also exhibited strong inhibition (97.80%) though not statistically significant, while the 100 mg/kg dose showed moderate inhibition (84.46%). These results indicate that *Z. americanus* water extract possesses significant peripheral analgesic activity, particularly at higher doses.

Table 4. Effects of Lempuyang Emprit Rhizome on glacial acetic acid test

Group	Dose	Mean ± SD (writhing)	Inhibition (%)	Significance vs Control
Negative Control	—	5836.00 ± 4633.07	—	—
Positive Control (Meloxicam 50 mg/kg)	2 mg/kg	80.67 ± 71.99	98.62	$p < 0.05$
Extract 100 mg/kg	100 mg/kg	906.67 ± 129.66	84.46	ns
Extract 200 mg/kg	200 mg/kg	128.33 ± 128.50	97.80	ns
Extract 300 mg/kg	300 mg/kg	66.33 ± 50.16	98.86	$p < 0.05$

Discussion

The analgesic activity study of lempuyang emprit Rhizome water extract was conducted using 25 male Wistar rats weighing approximately 200 g. Male rats were selected due to their relatively stable hormonal conditions and lower estrogen levels compared to female rats, which may reduce variability during the experiment. In addition, female rats are reported to exhibit higher stress levels that could potentially influence the test results (Lovick, 2021). Wistar rats were chosen as experimental animals because their anatomical, physiological, and genetic characteristics are considered comparable to those of humans, making them suitable for pharmacological studies.

Drug-Likeness Profile: Flavonoid Selectivity and Oral Potential

Of the 26 compounds identified by LC-HRMS, only six passed all five drug-likeness filters (Lipinski, Ghose, Veber, Egan, and Muegge). Notably, every compound that satisfied all criteria was either a flavonoid (alpinetin, pinostrobin, kaempferol, tetrahydroxychalcone, pinocembrin) or an amino-acid derivative (DL-tryptophan). This pattern is not accidental. The flavonoid scaffold inherently balances molecular weight (<500 Da), hydrogen-bond donors (<5), and lipophilicity (WLogP <5) — the very parameters the Lipinski rule targets. In contrast, highly polar amino acids such as arginine and asparagine, and bulky glycosides such as afzelin, failed the Ghose or Veber filters due to excessive polarity or rotatable-bond counts. These observations are consistent with a comprehensive review by Wang et al. (2017), who demonstrated that flavonoid aglycones preferentially satisfy oral drug-likeness criteria due to their balanced amphipathic character, whereas glycosylated flavonoids are systematically filtered by Veber's rotatable-bond and polar-surface-area thresholds.

Caspase-1 Docking: Binding Energies in Context

Alpinetin and (+)-pinocembrin each produced a binding energy of −4.6 kcal/mol against caspase-1 (PDB ID: 1RWK), surpassing the native ligand (−3.9 kcal/mol) by 0.7 kcal/mol 6. DL-tryptophan followed at −4.4 kcal/mol. All three compounds engaged the catalytic-site residues HIS237, ARG179, and CYS285 — the same triad that coordinates the native inhibitor's covalent interaction with caspase-1. This overlap in binding topography is mechanistically significant: it suggests that these flavonoids occupy the same substrate-binding pocket rather than binding to an allosteric site, raising the possibility of competitive inhibition of IL-1β maturation.

However, a binding-energy advantage of 0.7 kcal/mol over the native ligand must be interpreted cautiously. AutoDock Vina scores are estimates of gas-phase free energy

and do not account for solvation, entropic penalties from ligand desolvation, or the covalent-bond character of the native inhibitor. Priastari (2022) reported that pinostrobin — structurally related to pinocembrin — achieved −4.80 kcal/mol against the same target with identical residue contacts, a value only 0.2 kcal/mol lower than the present data for alpinetin and pinocembrin. This consistency across independent studies strengthens confidence in the binding pose, but the narrow energy range (−3.4 to −4.6 kcal/mol across all tested compounds) also indicates that the docking assay alone cannot rank inhibitor potency with high resolution.

The anti-inflammatory effects of *Zingiber* and *Alpinia* species have been extensively reviewed by Yuandani et al. (2023), who noted that gingerol, shogaol, and zerumbone suppress the NF-κB, COX-2, and LOX pathways, suggesting that the flavonoid compounds identified here may exert anti-inflammatory effects through parallel mechanisms beyond caspase-1 alone.

Yet a conceptual gap exists between these predictions and the in vivo protocol. The writhing test used oral gavage of the crude water extract, not purified flavonoids at their predicted intrinsic permeabilities. The extract matrix contains sugars, tannins, and other polar constituents that may retard flavonoid absorption through competitive dissolution or complexation. Serna González et al. (2022) demonstrated that pinostrobin — one of the flavonoids present in *Z. americanus* — exhibited limited oral bioavailability that could be substantially improved through β-cyclodextrin inclusion complexation, resulting in enhanced analgesic and anti-inflammatory effects. The present study's dose-dependent analgesia (84–99% inhibition across 100–300 mg/kg) suggests that sufficient flavonoid mass reached systemic circulation at the highest dose, but the crude extract dose required (300 mg/kg) is substantially higher than the purified-compound doses typically reported in comparable rodent models. This discrepancy between predicted HIA and the relatively high dose needed for efficacy warrants further pharmacokinetic characterisation and potentially the development of flavonoid-enriched formulations or inclusion complexes.

Analgesic Efficacy: Dose-Response Interpretation and Comparison with Meloxicam

The present study shows that the crude extract of *Z. americanus* produced a clear dose-dependent antinociceptive effect in the acetic acid writhing model, with the 300 mg/kg dose giving the strongest inhibition and nearly matching meloxicam. The response was not linear, because the effect rose sharply from 100 to 200 mg/kg and then plateaued at 300 mg/kg, which suggests that 200 mg/kg may already be close to the maximal effect in this assay (**Table 4**). The apparent separation between 200 and 300 mg/kg should be interpreted cautiously, since the 200 mg/kg group showed high variability and the overall significance was only marginal.

The dose-dependent pattern observed here is broadly consistent with previous studies on Zingiberaceae species. Sulaiman et al. (2009) demonstrated that the essential oil of *Zingiber zerumbet* at doses of 30, 100, and 300 mg/kg produced significant dose-dependent inhibition of acetic acid-induced abdominal writhing in mice, comparable to acetylsalicylic acid (100 mg/kg). Notably, their highest dose (300 mg/kg) matched the most effective dose in the present study, suggesting a consistent dose range for Zingiberaceae-derived analgesics. Chien et al. (2016) also reported that zerumbone, the major sesquiterpene from *Z. zerumbet*, significantly reduced the writhing response in mice at doses of 50–200 mg/kg and attenuated COX-2 expression through HO-1 induction.

Hossain et al. (2015) investigated germacrone, a cyclic sesquiterpene from *Curcuma aeruginosa* (also Zingiberaceae), and found that it produced 51.17% writhing inhibition at 40 mg/kg in the same model. The substantially higher inhibition achieved by *Z. americanus* extract (98.86% at 300 mg/kg) may reflect the synergistic contribution of multiple flavonoid compounds acting on different pain pathways simultaneously — an advantage that a single purified compound cannot match.

Choi et al. (2010) reported that ethanolic extracts of *Alpinia katsumadai* (a genus closely related to Zingiber and rich in alpinetin) at 30–300 mg/kg significantly inhibited phenylbenzoquinone-induced writhing in mice, with an IC_{50} of 0.044 μg/mL against COX-2 enzyme activity. The overlapping effective dose range (30–300 mg/kg) across multiple Zingiberaceae species supports the reproducibility of analgesic effects within this plant family.

Mechanistic Integration

The molecular docking identified caspase-1 inhibition as a plausible mechanism, with alpinetin and pinocembrin as lead candidates. The in vivo writhing test measures peripheral pain triggered by acetic-acid-induced prostaglandin release, a pathway that involves COX-2-mediated arachidonic-acid metabolism. Caspase-1 inhibition (targeting IL-1β maturation) and COX-2 inhibition (targeting prostaglandin synthesis) are distinct anti-inflammatory axes. The present study docked compounds against caspase-1 only, yet the *in vivo* efficacy may also involve COX-2 inhibition by the same flavonoids.

This mechanistic gap is important. Rudrapal et al. (2023) demonstrated through molecular docking, molecular dynamics, and in vitro enzyme assays that curcumin, gingerol, and capsaicin from Indian spices act as dual COX/5-LOX inhibitors, with curcumin exhibiting the best inhibitory activity against both COX-1/2 and 5-LOX enzymes. Given that alpinetin and pinocembrin share the flavonoid scaffold with gingerol, they may similarly engage multiple inflammatory targets. Sahoo et al. (2023) listed alpinetin among the polyphenolic compounds that target NF-κB and Nrf2 signalling pathways to reduce intestinal inflammation, further supporting a multi-target mechanism. More recently, Chen et al. (2025) identified a pinocembrin-derived galloyl-hexahydroxydiphenoyl glucoside (PINO) as a TRPV1 antagonist that stabilises the channel in a closed state, providing a non-opioid, ion-channel-targeted analgesic mechanism. PINO at 20 mg/kg reduced the writhing response in acetic acid-induced mice and elevated thermal and mechanical pain thresholds. This suggests that even if pinocembrin itself has modest caspase-1 affinity, its derivatives or structurally related flavonoids may act through TRPV1 antagonism — an entirely separate pathway from the caspase-1 mechanism proposed here.

Compared with recent studies, the present results are broadly consistent with work showing that pinostrobin-related structures become more active when their bioavailability or chemical structure is improved. González et al. (2022) reported that cyclodextrin complexation enhanced pinostrobin's anti-inflammatory and analgesic activity, which supports the present study's implication that formulation can strongly influence efficacy. González (2022) and Yuandani et al. (2023) also reviewed Zingiber and Alpinia species and concluded that their secondary metabolites act through immunomodulatory pathways, which aligns with the idea that the present extract may act through more than one inflammatory target. These studies converge with the current work by supporting flavonoid-type compounds as credible analgesic leads, but they also imply that crude extract activity may be limited by exposure, absorption, or matrix effects.

Advancing these agents will therefore require systematic screening against multiple inflammatory targets (COX-1, COX-2, TRPV1, caspase-1) and confirmation in pathway-specific in vitro assays. Suryadi et al. (2021) showed that pinostrobin — also present in *Z. americanus* — exhibited improved analgesic activity following acylation, with pinostrobin pentanoate achieving an ED_{50} of 10.37 mg/kg BW in the writhing test. This structure-activity relationship study underscores the potential for further optimisation of the lead flavonoids identified here.

Conclusion

Lempuyang emprit Rhizome water extract exhibited promising analgesic and anti-inflammatory activity. In silico analysis identified alpinetin and (+)-pinocembrin as the compounds with the highest affinity toward Caspase-1, while in vivo studies demonstrated dose-dependent analgesic effects comparable to meloxicam.

Nevertheless, this study has several limitations, including the use of computational predictions and animal models that may not fully represent human biological systems. In addition, alpinetin and (+)-pinocembrin were not isolated in pure form, and the bioavailability, pharmacokinetics, and toxicity profiles of the extract were not evaluated. Therefore, further studies are required to isolate the active compounds and confirm their pharmacological effects and safety.

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