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Research Article

Effect of Solvent Polarity and Protein Models on Anti-Inflammatory Properties of Kratom (*Mitragyna speciosa*) Leaf Extract

Nurul Muhlisa Mus[1], Vitryani Tandi Sole[2]*, Fajar Prasetya[3]

[1,3]Department of Pharmacology, Faculty of Pharmacy, Mulawarman University, Samarinda, East Borneo, Indonesia
[2]Pharmacy Study Program, Faculty of Pharmacy, Mulawarman University, Samarinda, East Borneo, Indonesia

*Correspondence email: tandisolevitryani@gmail.com

Abstract

Protein denaturation plays an important role in the inflammatory process, and inhibition of this mechanism is widely used as an indicator of anti-inflammatory potential in vitro. This study aimed to investigates the effect of solvent polarity and protein models on the anti-inflammatory activity of *Mitragyna speciosa* (kratom) leaf extract using albumin denaturation assays with egg albumin and bovine serum albumin (BSA). Extracts were prepared using solvents of different polarities—n-hexane (non-polar), ethyl acetate (semi-polar), and ethanol (polar)—and tested at concentrations of 200, 500, and 1000 ppm. The results showed that solvent polarity and assay model strongly influenced inhibitory activity. In the egg albumin model, the highest inhibition was observed in the ethyl acetate extract at 1000 ppm (45.35%) with an IC_{50} of 1232.5 ppm, followed by n-hexane extract (IC_{50} = 1966.7 ppm). Ethanol extract exhibited comparatively lower inhibition (maximum 27.84%; IC_{50} = 2214.7 ppm). Sodium diclofenac, used as a positive control, demonstrated potent activity with an IC_{50} of 249.3 ppm. In the BSA model, overall inhibition values increased across all extracts, with n-hexane and ethanolic extracts showing enhanced activity, reaching 75.10% and 73.94% inhibition at 1000 ppm, respectively. Ethanol extract demonstrated the strongest activity in the BSA model (IC_{50} = 281 ppm), indicating that polar constituents contribute substantially to protein-stabilizing effects. While, ethyl acetate extract displayed moderate inhibition with an IC_{50} of 697.6 ppm. The study highlights the importance of solvent selection and assay model in evaluating the bioactivity of kratom leaf extracts. Overall, kratom extracts possess measurable in vitro anti-inflammatory potential, though none surpassed the standard drug. Further phytochemical identification and in vivo studies are recommended to validate these activities.

Keywords: Kratom extract; protein denaturation; anti-inflammatory activity; solvent polarity

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1 Introduction

Inflammation serves as a fundamental biological response to tissue injury, infection, or other harmful stimuli, but when it becomes chronic, it contributes to pathologies such as arthritis, cardiovascular disease, and metabolic syndrome [1,2]. One of the most widely used *in vitro* approaches for evaluating the anti-inflammatory potential of natural extracts or isolated compounds is the protein denaturation inhibition assay, commonly performed using albumin such as bovine serum albumin (BSA) or egg albumin. Under stress conditions—such as exposure to heat—proteins may undergo denaturation, a process in which their three-dimensional structure becomes disrupted, leading to the loss of stability and biological function. Protein denaturation can expose new epitopes, enhance immunogenicity, and potentially trigger inflammatory responses [3]. Therefore, the ability to maintain protein structural integrity is considered relevant to modulating inflammatory processes at the molecular level.

The albumin denaturation assay is built upon this principle by assessing the capability of a test sample to prevent heat-induced structural alteration of albumin. Bioactive compounds that stabilize albumin under laboratory stress conditions are assumed to exhibit similar stabilizing effects within biological systems, thereby reducing protein-derived inflammatory triggers. Several phytopharmacological studies and methodological evaluations have reported that the inhibition of albumin denaturation correlates with anti-inflammatory activities observed in cell-based assays and animal models, supporting its use as a preliminary screening method before proceeding to more complex mechanistic investigations [5,15]. With advantages such as simplicity, rapid execution, sensitivity, and biological relevance to inflammation pathways, the protein denaturation inhibition assay has become an essential technique in early-stage screening of the anti-inflammatory potential of natural and synthetic compounds [6]. This approach also offers early insight into the capacity of test samples to preserve protein integrity—an important aspect in the regulation and attenuation of inflammatory responses at both the molecular and physiological levels [4].

Mitragyna speciosa, commonly known as kratom, is a tropical plant native to Southeast Asia, traditionally used for its stimulant, analgesic, and medicinal properties [7,8]. Its chemical profile is characterized by numerous bioactive constituents, particularly alkaloids such as mitragynine and 7-hydroxymitragynine, as well as phenolic compounds and flavonoids [8]. Modern pharmacological investigations have demonstrated that kratom extracts exhibit notable anti-inflammatory activities via multiple mechanisms: reduction of pro-inflammatory cytokines in lipopolysaccharide (LPS)-activated macrophages, and inhibition of inflammatory enzymes such as COX-2. In vivo studies — for example, carrageenan-induced paw edema in rodents — further support dose-dependent anti-inflammatory effects of kratom [9,10,11,12,13,33]. Nonetheless, despite these advances, there is a relative paucity of research leveraging in vitro protein denaturation assays to elucidate kratom's anti-inflammatory potential, particularly in relation to different solvent fractions.

The polarity of extraction solvents plays a critical role in shaping the phytochemical composition of plant extracts, which in turn influences their bioactivity. Non-polar solvents (e.g., n-hexane) preferentially extract lipophilic constituents such as terpenoids and sterols, while semi-polar solvents (e.g., ethyl acetate) and polar solvents (e.g., ethanol) are more effective in extracting phenolics, flavonoids, and polar alkaloids [14] —classes of compounds commonly associated with antioxidant and anti-inflammatory activities.

Against this background, the present study aims to evaluate and compare the in vitro anti-inflammatory potential of kratom leaf extracts derived using three solvents of varying polarity (n-hexane, ethyl acetate, ethanol) via two protein denaturation models: egg albumin and BSA. The anti-denaturation activity was quantified by determining the percentage inhibition and IC_{50} values.

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2 Method

2.1 Plant Materials, Extraction Procedure and Phytochemical Screening

Kratom leaves (*Mitragyna speciosa* Korth.) were collected from Kota Bangun, Kutai Kartanegara Regency, East Kalimantan, Indonesia, an area characterized by lowland tropical forest vegetation. Botanical identification of the plant material was conducted by the Herbarium Mulawarman, Ecology and Tropical Forest Biodiversity Conservation Laboratory, Faculty of Forestry, Mulawarman University, Samarinda, to ensure taxonomic accuracy. Kratom leaves were dried in an oven at 45 °C. The powdered leaves were then extracted using the maceration method with n-hexane, ethyl acetate, and 96% ethanol (1:10 w/v). The soaking process was carried out for 3×24 hours with periodic stirring. The obtained extracts were subjected to phytochemical screening to identify major classes of secondary metabolites, including alkaloids, flavonoids, steroids, tannins, and saponins.

2.2 In vitro Anti-inflammatory activity

a. Bovine Serum Albumin (BSA) Denaturation Assay

The anti-inflammatory potential of the extracts was evaluated using the protein denaturation inhibition assay, adapted with slight modifications from Ameena et al. (2023) [16]. Briefly, bovine serum albumin (BSA) was used as the model protein to assess the ability of samples to prevent heat-induced denaturation. A reaction mixture was prepared by combining 40 µL of BSA solution with 560 µL of phosphate-buffered saline (PBS, pH 6.4). Subsequently, 400 µL of sample solutions—representing the negative control, positive control, and extract fractions—were added at concentrations of 1000, 500, and 200 ppm, each prepared in triplicate. The mixtures were incubated at 37 °C for 20 minutes to allow interaction between protein and test compounds, followed by heating at 70 °C for 5 minutes to induce protein denaturation, as visualized by turbidity formation. After cooling to room temperature, 200 µL of each sample was transferred into a 96-well microplate, and the absorbance was recorded using an ELISA reader at 630 nm. The percentage inhibition of protein denaturation was calculated relative to the negative control.

b. Egg Albumin Denaturation Assay

The reaction mixture was prepared by adding 40 µL of egg albumin (from chicken egg), followed by 560 µL of Phosphate Bovine Saline (PBS, pH 6.4). Subsequently, 400 µL of the sample solution from the test control groups (negative control, positive control, and extract control) at concentrations of 1000 ppm, 500 ppm, and 200 ppm, each in triplicate, were added. The mixture was incubated at 37°C for 15 minutes, then heated at 70°C for 5 minutes to induce protein denaturation, indicated by the formation of turbidity. After cooling, the mixture was transferred into microplate wells and the absorbance was measured using an ELISA reader at 630 nm [16].

The percentage of protein denaturation inhibition was calculated using the following formula:

$$\%\text{Inhibition} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

In this equation, A_{control} represents the absorbance of the negative control (without extract), while A_{sample} refers to the absorbance obtained from the test sample or the positive control. A higher inhibition percentage indicates a stronger ability of the sample to prevent protein denaturation. The IC_{50} value, defined as the concentration required to achieve 50% inhibition, was determined by plotting sample concentration against the percentage inhibition.

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3 Result and Discussion

3.1 Extract yield and Phytochemical Screening

This study demonstrates that the type of solvent used in the extraction process significantly affects the yield of Kratom leaf extract (*Mitragyna speciosa* Korth). Among the three solvents tested, 96% ethanol produced the highest yield of 17.49%, which is much higher compared to ethyl acetate (4.71%) and n-hexane (2.4%). This indicates that polar solvents such as ethanol are more effective in extracting bioactive compounds from Kratom leaves compared to semi-polar (ethyl acetate) and non-polar (n-hexane) solvents. The solubility of polar and semi-polar compounds such as alkaloids, flavonoids, and phenolic compounds present in Kratom leaves is likely the main reason for the high yield of the ethanol extract. These compounds are known to possess potential pharmacological activities, including anti-inflammatory effects that have been previously studied. In contrast, non-polar solvents like n-hexane tend to extract components such as oils and fats, which are relatively small in amount in the leaf material, resulting in a lower yield.

Phytochemical screening of the n-hexane, ethyl acetate, and 96% ethanol extracts demonstrated distinct patterns of secondary metabolite distribution, reflecting the influence of solvent polarity on compound solubility (Table 1). Alkaloids were consistently detected in all extracts using Mayer, Dragendorff, and Wagner reagents, indicating their solubility across a range of polarities. Flavonoids and tannins showed a clear polarity-dependent trend, with the highest concentrations in the ethanol extract, moderate levels in ethyl acetate, and lowest in n-hexane, consistent with their polar nature and capacity for hydrogen bonding. Steroids were detected only in the non-polar n-hexane extract, confirming their lipophilic properties. Saponins were absent in n-hexane but present in ethyl acetate and ethanol extracts, reflecting their amphiphilic nature and preferential solubility in more polar solvents [34]. These results underscore the critical role of solvent polarity in determining the qualitative composition of secondary metabolites, which has direct implications for selecting extraction methods tailored to target bioactive compounds.

Table 1. Phytochemical Screening of n-Hexane, Ethyl Acetate, and 96% Ethanol Extracts

Secondary Metabolite	Reagent	n-Hexane Extract	Ethyl Acetate Extract	96% Ethanol Extract
Alkaloids	Mayer	+	+	+++
	Dragendorff	+	+	+++
	Wagner	+	+	+++
Flavonoids	Mg Powder + Concentrated HCl	+	++	+++
Steroids	Liebermann–Burchard	+	–	–
Tannins	1% $FeCl_3$	+	++	+++
Saponins	Hot Water + HCl	–	+	+++

+ = present / detected, ++ = moderate, +++ = high, – = absent / not detected

3.2 Anti-denaturation Activity in BSA Assay

The results of the protein denaturation assay (Table 2) using Bovine Serum Albumin (BSA) indicate that the anti-inflammatory activity of *Mitragyna speciosa* leaf extracts varies depending on the solvent polarity. Among the tested extracts, ethanol extract demonstrated the strongest activity, with an IC_{50} of 281.3 ppm, indicating that a relatively low concentration is sufficient to inhibit protein denaturation effectively. Although the maximum inhibition percentage of ethanol extract (73.94%) was slightly lower than that of n-hexane extract (75.10%), the lower IC_{50} value suggests a higher anti-inflammatory potential. This observation implies that polar compounds, such as flavonoids, phenolics, and polar

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alkaloids, extracted by ethanol, play a significant role in stabilizing BSA structure under denaturing conditions. Phenolic compounds interact with albumin via non-covalent bonds, stabilizing its tertiary structure [17,18, 19, 20, 21].

The n-hexane extract exhibited the highest inhibition percentage at 1000 ppm (75.10%), but its higher IC_{50} value (482.5 ppm) indicates that a higher concentration is needed to achieve a similar effect, reflecting a lower intrinsic anti-inflammatory potency compared to ethanol.This suggests that non-polar bioactive compounds, such as terpenoids or lipophilic constituents, contribute to the activity, but less effectively than polar compounds. N-hexane extract exhibited relatively higher activity with BSA than with egg albumin, suggesting that its non-polar components preferentially interact with the hydrophobic domains of BSA.

Ethyl acetate extract showed moderate activity, with an IC_{50} of 697.6 ppm and maximum inhibition of 58.12%. The intermediate activity of this semi-polar extract suggests that semi-polar compounds contribute to anti-inflammatory effects, though not as strongly as polar constituents.

Sodium diclofenac, used as a positive control, exhibited the highest inhibition (>93%) and an IC_{50} of less than 200 ppm, validating the assay and confirming that the extract activities are lower than standard pharmaceutical anti-inflammatory agents.

Overall, the findings indicate that ethanol extract demonstrates the most potent anti-inflammatory activity in the BSA assay, highlighting that polar solvents are more effective in extracting bioactive compounds capable of preventing protein denaturation.

3.3 Anti-denaturation Activity in Egg Albumin Assay

The egg albumin denaturation assay (Table 2) demonstrated that all kratom leaf extracts exhibited concentration-dependent inhibitory activity. As shown in Table 1, the n-hexane extract showed moderate inhibition (36.11–42.44%) with an IC_{50} of 1966.7 ppm, indicating relatively weak protection against heat-induced denaturation. The ethyl acetate extract exhibited improved activity (25.85–45.35%) with a lower IC_{50} (1232.5 ppm), while the ethanol extract showed the lowest inhibition (13.21–27.84%) and the highest IC_{50} (2214.7 ppm). Diclofenac sodium, used as the positive control, produced strong inhibition ranging from 49.63% to 55.42% with a significantly lower IC_{50} (249.3 ppm), confirming assay validity.

3.4 Comparative IC_{50} Across Solvents and Models

Bovine Serum Albumin (BSA) is a large animal serum protein with a molecular weight of approximately 66 kDa, composed of 583 amino acid residues that form a globular structure (Mdpi, 2016). Structurally, BSA consists of three main domains (I, II, III), each divided into two subdomains (A and B), arranged in such a way that they form a complex and stable folding, supported by 17 disulfide bridges [35]. This structure makes BSA fairly flexible while remaining stable against environmental changes, such as pH and temperature [24].

Ovalbumin (OVA) is the dominant protein in chicken egg white, accounting for approximately 54–58 % of total albumen protein, with a molecular weight of around 45 kDa and consisting of 386–388 amino acid residues [30]. This protein is a globular glycoprotein, combining α-helix and β-sheet secondary structures to form a complex tertiary fold. Its hydrophilic nature makes OVA water-soluble; however, the protein is sensitive to heat, extreme pH, or mechanical stress, which can lead to denaturation, aggregation, and loss of biological functions such as gelation or foaming [31]. Due to its susceptibility to denaturation, ovalbumin is frequently used as a model protein in in vitro anti-denaturation or anti-inflammatory assays. The principle of these assays is based on the ability of a bioactive compound or extract to maintain OVA's native conformation under heat or chemical stress. Compounds that can preserve solubility or prevent coagulation of OVA demonstrate protective activity toward proteins, which may correlate with anti-inflammatory or antioxidant potential. This methodology

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provides a simple, reproducible, and biologically relevant screening system to evaluate protein stabilization effects of bioactive compounds [32].

A cross-comparison of IC_{50} values revealed notable differences in the sensitivity of the two protein-denaturation models. The BSA assay proved more responsive than the egg albumin assay in detecting anti-denaturation effects, as reflected by consistently lower IC_{50} values across all extracts (Table 2). Thermal denaturation of egg-white ovalbumin has been shown to proceed via irreversible first-order kinetics, leading to rapid conversion of the native protein into aggregated or gelated forms [22].Once the native tertiary structure is lost and aggregates form, binding sites for small stabilizing ligands (such as polyphenols or flavonoids) are no longer available, so the stabilizing compounds cannot effectively or consistently prevent denaturation. Furthermore, aggregation introduces physical heterogeneity (size, oligomeric structure), causing measurement signals (e.g., UV absorbance, viscosity, or density) to vary significantly between experiments. Therefore, the protective response of stabilizers toward proteins like ovalbumin tends to be inconsistent and less reproducible. In contrast, Bovine Serum Albumin (BSA) has been shown to retain part of its native structure upon heating and to undergo unfolding and aggregation in kinetically separable stages, allowing structural changes — as well as ligand-induced protective effects — to be monitored and quantified reproducibly [23]. Consequently, BSA provides a more reliable and sensitive model for evaluating the anti-denaturation effects of phytochemical extracts or other bioactive compounds compared to egg albumin.

Among the tested samples (table 2), the ethanol extract demonstrated the strongest activity in the BSA model (IC_{50}=281 ppm), indicating that polar constituents contribute substantially to protein-stabilizing effects. Polar compounds, such as phenolics and flavonoids, can interact with Bovine Serum Albumin (BSA) through hydrogen bonding and electrostatic interactions with polar or charged residues on the protein surface, thereby helping to stabilize the three-dimensional structure of BSA. In studies on phenolic acids and BSA, these compounds were found to form hydrogen bonds with specific amino acid residues (e.g., Thr, Lys, Asn), along with hydrophobic and electrostatic interactions, collectively maintaining the native conformation of BSA even under stress conditions [28]. Other studies using plant extracts have shown that total phenolic and flavonoid content correlates with the extract's ability to inhibit BSA denaturation, indicating that these polar compounds can effectively protect the protein from heat-induced unfolding [29]. This protective mechanism involves stabilizing internal noncovalent interactions and forming a "hydration shell" around the protein, allowing the BSA structure to remain intact despite denaturing treatments. The ethyl acetate extract exhibited intermediate potency, which aligns with the nature of its semipolar phytochemicals that offer a balanced profile between polarity and lipophilicity.

In contrast, the nonpolar n-hexane extract exhibited the lowest activity in the egg albumin assay, while showing moderate inhibition in the BSA assay, indicating that certain lipophilic compounds may preferentially bind to serum albumin. The interaction between nonpolar compounds and BSA plays a critical role in enhancing protein stability against denaturation. Nonpolar molecules inherently avoid aqueous environments and tend to associate with the protein's hydrophobic regions. By occupying these pockets, nonpolar compounds limit excessive flexibility within the protein fold, preserve internal noncovalent interactions, and prevent unfolding or aggregation induced by heat or harsh solvents [24]. This explains why extracts rich in nonpolar constituents can improve the stability of BSA under denaturing conditions. Overall, the three-dimensional architecture of BSA, featuring globular domains and concealed hydrophobic sites, enables it to act as a "host" for various nonpolar molecules, making these interactions specific, stable, and biologically meaningful, both for the transport of lipophilic ligands and for evaluating anti-denaturation activity [24,25,26,27].

Collectively, these findings underscore the importance of solvent selection and assay model in evaluating the bioactivity of kratom leaf extracts.

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Table 2. Comparison of Anti-Inflammatory Effects of Kratom Extracts Prepared with Different Solvent Polarities Using Egg Albumin and BSA Models

Method	Sampel	Concentration (ppm)	Mean Absorbance ± SD	% Inhibition	IC_{50}
Egg Albumin	Negative Control	-	1.075 ± 0.026	-	-
	N-Hexane	200	0.687 ± 0.061	36.11	1966.7
		500	0.663 ± 0.138	38.34	
		1000	0.619 ± 0.120	42.44	
	Ethyl Asetate	200	0.797 ± 0.042	25.85	1232.5
		500	0.773 ± 0.224	28.08	
		1000	0.588 ± 0.195	45.35	
	Ethanol	200	0.933 ± 0.035	13.21	2214.7
		500	0.850 ± 0.037	20.99	
		1000	0.776 ± 0.052	27.84	
	Sodium Diclofenac	200	0.542 ± 0.031	49.63	249.3
		500	0.518 ± 0.032	51.86	
		1000	0.479 ± 0.026	55.42	
BSA	Negative Control	-	1.035 ± 0.049	-	-
	N-Hexane	200	0.726 ± 0.235	29.86	482.5
		500	0.427 ± 0.200	58.73	
		1000	0.258 ± 0.167	75.10	
	Ethyl Asetate	200	0.690 ± 0.393	33.25	697.6
		500	0.552 ± 0.263	46.65	
		1000	0.433 ± 0.106	58.12	
	Ethanol	200	0.580 ± 0.045	43.91	281.3
		500	0.383 ± 0.105	62.95	
		1000	0.270 ± 0.047	73.94	
	Sodium Diclofenac	200	0.070 ± 0.037	93.27	<200
		500	0.065 ± 0.017	93.72	
		1000	0.060 ± 0.009	94.23	

4 Conclusion

The research results indicate that solvents with different polarity levels significantly affect the anti-inflammatory activity of Kratom leaf extract. Extracts obtained using non-polar (N-Hexane) and semi-polar (Ethyl Acetate) solvents exhibited different inhibitory abilities against protein models (Egg Albumin and BSA). The use of the BSA protein model showed a higher inhibition rate compared to Egg Albumin, especially at increasing extract concentrations. Furthermore, the lower IC_{50} value in BSA compared to Egg Albumin indicates a more effective anti-inflammatory potential of the Kratom extract in the BSA protein model. Therefore, solvent polarity and protein model type greatly influence the measurement of the anti-inflammatory activity of Kratom leaf extract. Based on these results, it is recommended to conduct further fractionation, quantitative phytochemical analysis, and in vivo testing to validate the extract's potential. Practically, this study implies that the choice of solvent is crucial for extract effectiveness, the BSA model is suitable as a standard evaluation method, and Kratom leaves have potential as a natural anti-inflammatory candidate for further research and herbal product development.

5 Declarations

5.1 Author contributions

All authors contributed significantly to this study. Nurul Muhlisa Mus conceptualized and designed the research, performed statistical analyses, interpreted the results and drafted the initial manuscript. Vitryani Tandi Sole carried out the laboratory experiments, sample preparation, and anti-inflammatory activity assays. Fajar Prasetya contributed to the discussion and conclusion sections. All authors have read and approved the final version of the manuscript.

5.2 Conflict of Interest

The authors declare that they have no known competing financial or personal relationships that could have appeared to influence the work reported in this study.

5.3 Funding Statement

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