RBRU International Journal of Science and Technology 1 (1) (2026) 13–22



RBRU International Journal of Science and Technology

ISSN: 3088-389X (online) journal homepage: https://ph05.tci-thaijo.org/index.php/rbruijst



Research Article

Development and Performance Evaluation of a Rapid Microplate-Based Bovine Serum Albumin Protein Denaturation Assay for Anti-Inflammatory Screening

Maneenuch Pengsawang[a], Sunisa Suwancharoen[b,c,*]

[a] *Faculty of Science, Department of Chemistry, Kasetsart University, Chatuchak, Bangkok 10903, Thailand*
[b] *Herbal Product for Health and Beauty Research and Development Center, Rambhai Barni Rajabhat University, Chanthaburi 22000, Thailand*
[c] *Department of Chemistry, Faculty of Science and Technology, Rambhai Barni Rajabhat University, Chanthaburi 22000, Thailand*

ARTICLE INFO

Keywords:
Anti-inflammatory activity
Bovine serum albumin
Microplate assay
Protein Denaturation

Article history:
Received 5 August 2026
Revised 27 August 2026
Accepted 30 August 2026
Published 31 August 2026

ABSTRACT

The bovine serum albumin (BSA) protein denaturation assay is one of the most widely used in vitro methods for preliminary screening of anti-inflammatory activity because of its simplicity and low cost. However, the conventional tube-based assay requires relatively large reagent volumes, prolonged analytical time, and intensive manual handling, limiting its application for high-throughput screening. Therefore, the development of a rapid and reagent-saving analytical platform with comparable analytical performance is highly desirable. This study aimed to develop and evaluate a microplate-based BSA protein denaturation assay for anti-inflammatory screening. The conventional protocol was adapted to a 96-well microplate format by optimizing the experimental conditions. The main optimized conditions were determined to be 0.7% (w/v) BSA, a 150:50 µL BSA-to-methanol ratio, heating at 72°C for 4 min, and no room temperature pre-incubation. Diclofenac sodium was employed as the reference anti-inflammatory drug to compare the analytical performance of the developed method with the conventional assay. The applicability of the proposed method was further demonstrated using ethanolic extracts of *Zingiber montanum* and *Etlingera pavieana.* No statistically significant difference was detected between the IC_{50} values obtained under the two assay conditions (p>0.05). In addition, the developed method reduced the total reaction volume 15-fold and shortened the analytical time. The assay was successfully applied to evaluate the anti-inflammatory activities of the selected medicinal plant extracts, demonstrating its applicability for routine laboratory analysis. The developed microplate-based BSA protein denaturation assay provides a rapid, reliable, and cost-effective alternative to the conventional tube-based method. The proposed protocol is suitable for routine and high-throughput preliminary screening of anti-inflammatory compounds and offers an efficient analytical platform for natural product research.

*Corresponding author
DOI:10.68654/rbruijst.2026.01.02
maneenuch.pe@gmail.com (M. Pengsawang); sunisa.s@rbru.ac.th (S. Suwancharoen)
This is an open access article under the Creative Commons Attribution – NonCommercial – NoDerivatives 4.0 license.
© 2026 RBRU International Journal of Science and Technology – Published by Rambhai Barni Rajabhat University.

1. Introduction

Inflammation is a complex biological response initiated by the immune system to protect the body against infections, tissue injury, and other harmful stimuli [1, 2]. Under normal physiological conditions, the inflammatory response promotes the elimination of pathogens and damaged tissues, followed by tissue repair and restoration of homeostasis [1]. However, persistent or dysregulated inflammation has been recognized as a major contributing factor to the pathogenesis of numerous chronic diseases, including rheumatoid arthritis, inflammatory bowel disease, cardiovascular disease, diabetes mellitus, neurodegenerative disorders, and several types of cancer [2–4]. Consequently, the discovery and development of effective anti-inflammatory agents remain an important research focus in pharmaceutical sciences, medicinal chemistry, and natural product research [4, 5].

The evaluation of anti-inflammatory activity can be performed using both *in vivo* and *in vitro* approaches. Although animal models provide valuable physiological information, they are generally associated with ethical concerns, high experimental costs, and relatively long analysis times [6, 7]. Therefore, *in vitro* screening methods have become increasingly important for the preliminary evaluation of anti-inflammatory activity because they are rapid, inexpensive, reproducible, and suitable for screening large numbers of samples [6–10]. Among these methods, the bovine serum albumin (BSA) protein denaturation assay is one of the most widely employed techniques for preliminary anti-inflammatory screening. The assay is based on the principle that many inflammatory conditions are associated with protein denaturation, and compounds capable of preventing heat-induced protein denaturation may exhibit anti-inflammatory property [7, 9, 11–13]. Owing to its simplicity, low operational cost, and satisfactory reproducibility [13], the BSA protein denaturation assay has been extensively applied for evaluating the anti-inflammatory potential of synthetic compounds, medicinal plants, and natural product [7, 8, 11, 15].

Despite its widespread application, the conventional BSA protein denaturation assay is generally performed using test tubes, requiring relatively large reaction volumes, considerable reagent consumption, and multiple manual handling steps [9, 11, 15]. These limitations increase analytical cost and experimental time while reducing sample throughput, particularly when large numbers of samples are analyzed [16–18]. In recent years, microplate-based analytical platforms have gained increasing attention in biochemical and pharmaceutical analyses due to their ability to reduce reagent consumption, shorten analysis time, and facilitate high-throughput screening [16–19]. Recent studies have also reported the use of the BSA protein denaturation assay in a 96-well microplate format for preliminary anti-inflammatory screening [20]. However, compared with conventional tube-based approaches, a systematically optimized microplate-based BSA protein denaturation assay with comprehensive analytical performance evaluation remains limited. Therefore, the development of a simple, reliable, and cost-effective microplate-based protocol capable of providing analytical performance comparable to the conventional tube-based assay remains necessary.

Medicinal plant extracts are frequently employed as representative samples for evaluating newly developed screening methods because they contain diverse bioactive constituents with potential anti-inflammatory activity [5, 7, 8, 21]. Several studies have reported the anti-inflammatory potential of plant-derived materials and natural product-based formulations using *in vitro* evaluation approaches, including inhibition of protein denaturation assays [13, 22–24]. In the present study, ethanolic extracts of *Zingiber montanum* and *Etlingera pavieana* were selected solely as representative plant samples to demonstrate the applicability of the developed microplate-based assay rather than to investigate their pharmacological activities [25–27].

Therefore, this study aimed to develop and optimize a microplate-based BSA protein denaturation assay for preliminary anti-inflammatory screening. The analytical performance of the proposed method was evaluated by comparison with the conventional tube-based assay using diclofenac sodium as the reference standard. The practical applicability of the developed method was subsequently demonstrated using ethanolic extracts of *Zingiber montanum* and *Etlingera pavieana* as representative plant samples. The proposed assay is expected to reduce reagent consumption and analytical time while maintaining analytical performance comparable to the conventional method, thereby providing an efficient platform for routine laboratory applications and high-throughput screening of anti-inflammatory compounds.

2. Materials and Methods

2.1. Chemicals and reagents

Bovine serum albumin (BSA; analytical grade, EMD Millipore Corp., USA), Tris (hydroxymethyl)aminomethane (Tris; laboratory grade, OmniPur, Germany), glacial acetic acid (analytical grade, Chaemikit Limited Partnership,

Thailand), methanol (HPLC grade), and diclofenac sodium (pharmacopoeial grade, A-Fenac, Thailand) were used throughout the study without further purification. Ultrapure water was used for the preparation of all solutions.

Absorbance measurements were performed using a UV-Visible spectrophotometer (Genesys 10S, Thermo Scientific, USA) and a 96-well microplate reader (M965+, Metertech, Taiwan). Incubation of reaction mixtures was carried out using a thermostatically controlled water bath (Memmert, Germany) and a dry block heater (OHAUS, Switzerland).

2.2. Plant materials

Samples of dried rhizomes of *Zingiber montanum* and *Etlingera pavieana* were collected from Ban Thap Sai Lang, Pong Nam Ron District, Chanthaburi Province, Thailand. The botanical identities of the plant materials were verified by the authors based on taxonomic keys. Ethanolic extracts were prepared by macerating air-dried rhizomes in 95% ethanol at a ratio of 1:2 (w/v) for 24 hours. After filtration, the solvent was removed by rotary vacuum evaporation to obtain crude extracts, which were used as representative plant samples to demonstrate the applicability of the developed microplate-based bovine serum albumin (BSA) protein denaturation assay.

2.3. Preparation of sample solutions

A 0.05 M Tris-acetate buffer (pH 6.8) was prepared and used as the solvent for BSA solution preparation. The pH of the buffer was adjusted using 0.1 M acetic acid and 0.1 M sodium hydroxide. BSA solutions at concentrations of 0.2, 0.5, 0.7, and 1.0% (w/v) were prepared by dissolving an appropriate amount of BSA in Tris-acetate buffer (pH 6.8). Diclofenac sodium was prepared in methanol as a reference standard at a stock concentration of 1000 µg/mL and subsequently diluted to the required concentrations (10-100 µg/mL. The ethanolic plant extracts of *Zingiber montanum* and *Etlingera pavieana* were dissolved in methanol to prepare stock solutions at a concentration of 1000 µg/mL. The stock solutions were further diluted with methanol to obtain working concentrations of 5, 10, 20, and 100 µg/mL for evaluation using the BSA protein denaturation assay.

2.4. Conventional BSA protein denaturation assay

The conventional BSA protein denaturation assay was performed according to a previously reported method [21] with slight modifications. Briefly, the reaction mixture consisted of 150 mL of diclofenac sodium or plant extract solution and 2,850 mL of 0.2% (w/v) BSA solution prepared in 0.05 M Tris-acetate buffer (pH 6.8). The reaction mixtures were incubated at room temperature for 15 min, heated at 72°C for 4 min, and subsequently cooled at room temperature for 20 min. The absorbance of each reaction mixture was measured at 660 nm using a UV-Vis spectrophotometer. Diclofenac sodium was used as the reference standard. The experiment was independently performed 12 times (n=12).

2.5. Development and optimization of the microplate method

The conventional BSA protein denaturation assay reported by Nirmal and Panichayupakaranant [21] was adapted to a 96-well microplate format to reduce reagent consumption and improve analytical throughput. Method optimization was performed by systematically evaluating critical experimental parameters within specific ranges, including BSA concentration (0.2, 0.5, 0.7, and 1.0% w/v), BSA-to-methanol ratio (190:10, 170:30, 160:40, and 150:50 µL, heating time at 72°C (3 and 4 min), and the inclusion or omission of a 15 min room temperature pre-incubation step, while maintaining all other experimental variables constant. The optimum conditions were selected based on assay sensitivity, repeatability, and the ability to produce a stable absorbance signal with minimal background interference.

The optimized assay was carried out in a flat-bottom 96-well microplate. BSA solution (0.7% w/v) prepared in Tris-acetate buffer (pH 6.8) was mixed with the test sample or diclofenac (positive control) at the optimized reaction volume. The reaction mixture was incubated under the optimized conditions and subsequently heated at 72°C for 4 min to induce protein denaturation. After heating, the plate was cooled at room temperature for 10 min, and the absorbance was measured at 650 nm using a microplate reader. To systematically control for potential solvent effects and sample background, the “Control” was prepared using 150 µL of 0.7% (w/v) BSA solution and 50 µL of pure methanol (without test compounds). The “Blank” (or sample blank) for each reaction was prepared using 150 µL of Tris-acetate buffer and 50 µL of the respective test sample or standard dissolved in methanol (without BSA protein). Both control and sample mixtures underwent the identical heating procedure at 72°C for 4 min to ensure consistent experimental treatment. For method validation using the reference standard (diclofenac sodium), each concentration was analyzed in 12 independent replicates (n=12).

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

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

where the experimental components are prepared and defined as follows:
A_{control}: Consists of 150 µL of 0.7% (w/v) BSA solution in Tris-acetate buffer and 50 µL of pure methanol solvent (without test compounds). This reaction undergoes the identical heating and cooling procedures to establish the maximum (100%) protein denaturation baseline.
A_{sample}: Consists of 150 µL of 0.7% (w/v) BSA solution in Tris-acetate buffer and 50 µL of plant extract solution (5, 10, or 20 µg/mL) or reference standard prepared in methanol, subjected to the heating and cooling steps.

$A_{\text{sample blank}}$: Consists of 150 µL of Tris-acetate buffer (pH 6.8, without BSA) and 50 µL of the respective plant extract or reference standard solution. It undergoes the same heating and cooling treatment to correct for the intrinsic color, turbidity, or background absorbance contributed by the plant extracts.

$A_{\text{control blank}}$: Consists of 150 µL of Tris-acetate buffer (without BSA) and 50 µL of pure methanol. This is used to calibrate and subtract the baseline absorbance of the well plate and solvent background.

$A_{\text{positive control}}$: Consists of 150 µL of 0.7% (w/v) BSA solution and 50 µL of diclofenac sodium in methanol (10-100 µg/mL), representing the reference anti-inflammatory drug treated under the same experimental conditions.

2.6. Statistical analysis

Each concentration was analyzed in triplicate (three technical replicates) within each experiment. To ensure statistical validity, the experiments were independently repeated 12 times for the reference standard (yielding 12 independent IC_{50} estimates, n=12) and 3 times for the plant extracts (yielding 3 independent IC_{50} estimates, n=3). Measurements from triplicate wells on the same plate were averaged to serve as a single data point for each independent run and were never treated as independent replicates. Outliers were identified using Dixons Q test at a 95% confidence level (alpha = 0.05) and excluded prior to analysis. The concentration-response curves were constructed, and the IC_{50} values were estimated by fitting the data to a nonlinear regression model using a four-parameter logistic curve (sigmoidal dose-response with variable slope) in GraphPad Prism. The linear regression analysis described in Section 3.2 was employed solely to evaluate the linear trend of concentration dependent inhibition within the tested range, not for IC_{50} estimation. Statistical significance between IC_{50} values of the two methods was evaluated using an unpaired, two-tailed Students t-test. A p-value of < 0.05 was considered statistically significant

3. Results and Discussions

3.1. Optimization of the microplate-based BSA protein denaturation assay

To establish a rapid and reliable microplate-based BSA protein denaturation assay, the experimental conditions were systematically optimized by evaluating the effects of BSA concentration, BSA-to-methanol ratio, heating time, and room-temperature incubation before heating (Figure 1).

3.1.1. Effect of BSA concentration and BSA-to-methanol ratio

As shown in Figure 1(a), the assay conditions reported for the conventional tube-based method could not be directly applied to the microplate format because no detectable protein precipitation was observed when 0.2% (w/v) BSA was used, regardless of the BSA-to-methanol ratio. Increasing the BSA concentration enhanced protein precipitation, as indicated by the increase in absorbance at 650 nm. At 0.5% (w/v) BSA, protein precipitation was first observed at a BSA-to-methanol ratio of 150:50 µL. Higher absorbance values were obtained using 0.7% and 1.0% (w/v) BSA, particularly at the 150:50 µL ratio. However, the 1.0% BSA solution produced excessive turbidity, which may interfere with absorbance measurement and reduce analytical reproducibility. Therefore, 0.7% (w/v) BSA with a reaction composition of 150 µL BSA solution and 50 µL methanol was selected as the optimal condition because it generated a clear protein precipitation signal while minimizing background turbidity.

3.1.2. Effect of heating time

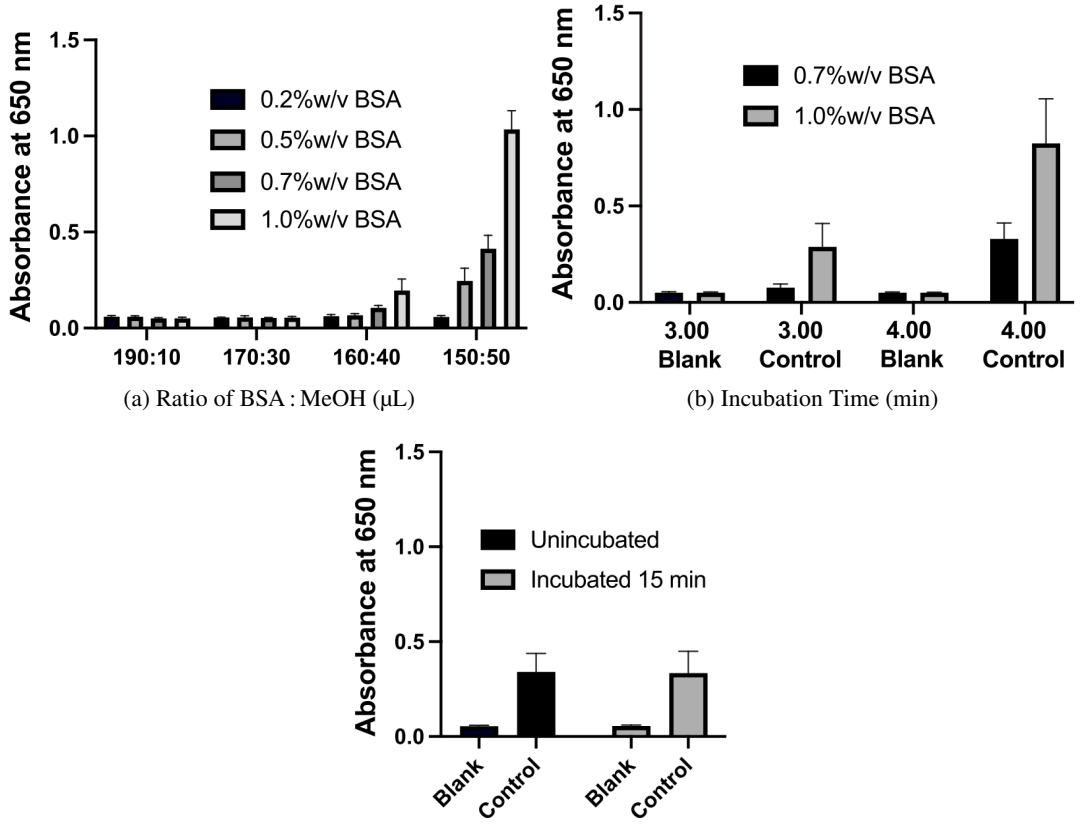
The effect of heating time was subsequently investigated using the selected reaction composition (Figure 1(b)). Heating at 72°C for 3 min was insufficient to induce protein denaturation when 0.7% BSA was used, whereas heating for 4 min produced a marked increase in absorbance, indicating effective protein precipitation. Although both incubation times generated measurable precipitation with 1.0% BSA, the higher protein concentration was not selected because of excessive turbidity. Therefore, a heating time of 4 min was chosen for the optimized assay.

3.1.3. Effect of room temperature incubation

The influence of room-temperature incubation before heating was further evaluated (Figure 1(c)). Both 0.7% and 1.0% (w/v) BSA concentrations were included in this evaluation to confirm that the effect of omitting the pre-incubation step was consistent and did not alter protein denaturation kinetics across different protein levels that yielded detectable precipitation. Comparable absorbance values were obtained in the control reactions regardless of whether the samples were incubated at room temperature for 15 min prior to heating. Since the omission of this incubation step did not noticeably affect protein precipitation at either concentration, the pre-incubation step was excluded from the final protocol. This modification shortened the assay time by approximately 15 min while maintaining comparable analytical performance.

3.1.4. Summary of optimized conditions

Based on the optimization results, the final assay conditions consisted of 0.7% (w/v) BSA, a 150:50 µL BSA-to-methanol ratio, heating at 72°C for 4 min, and no room-temperature pre-incubation (Table 1). These conditions provided adequate protein precipitation, minimized background turbidity, reduced assay time, and were subsequently employed for all anti-inflammatory activity determinations.



(a) Ratio of BSA : MeOH (µL)

(b) Incubation Time (min)

(c) Incubation Condition

Figure 1: Optimization of experimental conditions for anti-inflammatory assay. (a) Effect of BSA concentrations and BSA : MeOH ratios (µL) on absorbance at 650 nm. (b) Effect of incubation time (3 and 4 min) at 0.7% and 1.0% w/v BSA. (c) Effect of additional incubation at room temperature (15 min) compared with unincubated condition. Data are expressed as mean ±SD (n=12).

Table 1
Summary of optimal condition.

Parameter	Optimal condition
BSA concentration	0.7% w/v
Ratio BSA : MeOH	150:50 µL
Incubation Time	4 min
Additional Incubation	Unincubated

3.2. Comparison of analytical performance between the microplate and conventional tube-based assays

Figure 2 compares the anti-inflammatory activity of diclofenac sodium determined by the conventional tube-based and the developed microplate BSA protein denaturation assays. For both methods, the percentage inhibition increased in a concentration-dependent manner over the tested concentration range (10-100 µg/mL). While the complete concentration-response curve over a wider range typically follows a sigmoidal nonlinear profile, the selected tested range (10-100 µg/mL) falls within the dynamic linear region of the assay, where the inhibition rate is highly proportional to the concentration. Consequently, linear regression analysis showed excellent linearity for both methods, with coefficients of determination (R^2) of 0.9876 and 0.9875, respectively. The estimated IC_{50} values were 40.63 ± 6.85 µg/mL for the conventional assay and 43.15 ± 7.99 µg/mL for the developed microplate assay. Statistical comparison using an unpaired Students t-test confirmed that no statistically significant difference was detected between the IC_{50} values of the two methods ($p = 0.415$). The 3.5-fold increase in BSA concentration (from 0.2% w/v in the conventional assay to 0.7% w/v in the microplate assay) was methodologically necessary because the shorter optical pathlength of the 96-well microplate format, compared to a standard 10 mm spectrophotometric cuvette, resulted in no detectable turbidity signal at 0.2% BSA. Mechanistically, changes in protein concentration can influence the thermodynamic stability of BSA, the kinetics of heat-induced unfolding, and the binding equilibrium between the protein and the test compounds 2. Despite these different reaction conditions, the close agreement of the calculated IC_{50} values can be justified because the percentage inhibition is normalized relative to each systems respective maximum denaturation control. Furthermore, the binding of NSAIDs like diclofenac to specific hydrophobic cavities on BSA (such as Sudlows sites I and II) to prevent thermal unfolding remains highly efficient within this range 34. However, because the alteration of protein to drug ratios can modify molecular binding partitioning, these two assays cannot be considered strictly equivalent analytically, but are highly comparable for preliminary screening purposes.

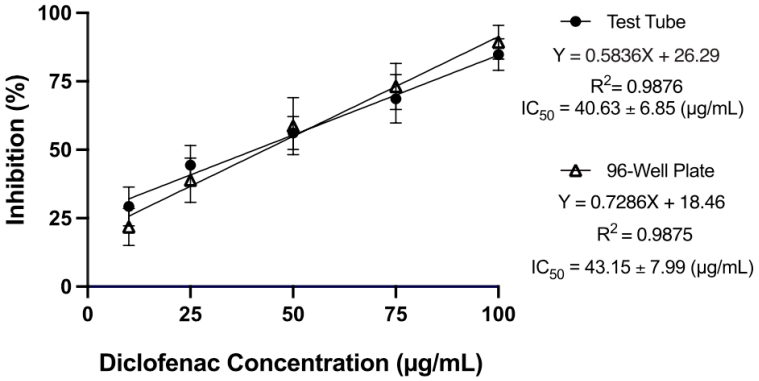


Figure 2: Inhibitory effect of diclofenac sodium on protein denaturation. Comparison of anti-inflammatory activity evaluated using the conventional test tube method (•) and the 96-well plate microplate assay (△) at various diclofenac concentrations (10-100 µg/mL). Linear regression equations, coefficients of determination (R^2), and IC_{50} values (µg/mL) for both methods are shown. Data are expressed as mean ± SD (n=12).

3.3. Comparison of reagent consumption, analysis time, and experimental cost

The analytical performance of the developed microplate assay was further evaluated by comparing its operational characteristics with those of the conventional tube-based method reported by Nirmal and Panichayupakaranant [21] (Table 2). The optimized microplate protocol employed 0.7% (w/v) BSA, whereas the conventional assay used 0.2% (w/v) BSA. Although a higher BSA concentration was required to achieve adequate protein precipitation in the reduced reaction volume, the total reaction volume was markedly decreased 15-fold from 3,000 µL to 200 µL. In addition to reducing the total reaction volume, the optimized protocol simplified the analytical procedure by eliminating the 15-min room temperature pre-incubation step and reducing the post-heating cooling time from 20 min to 10 min. Consequently, the total assay time was reduced from 39 min to 14 min, representing approximately a 2.8-fold decrease in analysis time.

The experimental cost was evaluated quantitatively based on the consumption of key reagents and disposable materials. Specifically, the required mass of BSA protein per reaction was reduced by approximately 5.4-fold (from 5.7 mg in the conventional assay to 1.05 mg in the optimized assay), while the sample solution volume was decreased 3-fold (from 150 µL to 50 µL), and the Tris-acetate buffer solvent volume was reduced 19-fold (from 2,850 µL to 150 µL). Overall, the total material cost per single reaction was estimated to be reduced by approximately 85%. This quantitative cost reduction was driven by both the savings in BSA protein consumption and the substitution of individual disposable semi-micro spectrophotometric cuvettes with a single flat-bottom 96-well microplate, thereby substantially minimizing consumable expenses.

Another important advantage of the developed method is its substantially higher analytical throughput. The conventional tube-based assay requires individual measurement of each reaction using a UV-Visible spectrophotometer, thereby limiting sample throughput. In contrast, the developed microplate assay allows simultaneous measurement of up to 96 samples within approximately 1 min using a microplate reader. This feature considerably increases sample throughput while improving laboratory efficiency, making the proposed method particularly suitable for routine screening and high-throughput evaluation of anti-inflammatory compounds.

Overall, the developed microplate assay substantially reduced the total reaction volume and analysis time while maintaining analytical performance comparable to that of the conventional tube-based method. These advantages make the proposed assay a practical alternative for routine laboratory applications and large-scale preliminary screening of anti-inflammatory agents. Furthermore, the reduction in sample volume from 150 µL to 50 µL offers a highly practical advantage, particularly in natural product research where bioactive plant extracts are often available only in limited quantities. This minimized sample requirement ensures that valuable materials are preserved for further downstream characterization. Additionally, the satisfactory reproducibility of the developed microplate method was demonstrated by the consistent percentage inhibition values and the low variation in IC_{50} estimates obtained across 12 independent experimental runs (n=12), confirming the reliability of this optimized platform for routine anti-inflammatory screening.

Table 2
Comparison of the conventional and optimized 96-well microplate protein denaturation assays.

Parameter	Conventional	Optimized
Total reaction volume (µL)	3000	200
BSA concentration (% w/v)	0.2	0.7
BSA : MeOH ratio (µL)	2850:150	150:50
Sample volume (µL)	150	50
Heating time 72°C (min)	4	4
Cooling time (min)	20	10
Additional incubation at room temperature (min)	15	None
Detection wavelength (nm)	660	650
Total assay time (min)	39	14

3.4. Application of the developed microplate assay to the evaluation of plant extracts

The applicability of the developed microplate-based BSA protein denaturation assay was evaluated using ethanolic extracts of *Zingiber montanum* and *Etlingera pavieana* as representative plant samples. As shown in Table 3, both extracts exhibited concentration-dependent inhibition of heat-induced protein denaturation over the tested concentration range (5-20 µg/mL). The percentage inhibition increased with increasing extract concentration, indicating that the developed assay was capable of detecting differences in anti-inflammatory activity among the tested samples. The ethanolic extract of *Zingiber montanum* showed inhibition values of 22.93±4.66%, 45.09±3.73%, and 60.78±6.29% at concentrations of 5, 10, and 20 µg/mL, respectively, with an IC_{50} value of 13.31 ± 3.00 µg/mL. Similarly, the ethanolic extract of *Etlingera pavieana* exhibited inhibition values of 23.41±4.94%, 41.74± 6.44% and 64.53±5.56%, corresponding to an IC_{50} value of 14.31±0.79 µg/mL.

Statistical analysis demonstrated that the IC_{50} values of the two plant extracts were not significantly different (p>0.05), indicating comparable anti-inflammatory activities under the experimental conditions employed. These findings demonstrate that the developed microplate assay is suitable for the preliminary evaluation of anti-inflammatory activity in plant extracts and can be applied as a rapid and reagent-efficient screening platform for natural products. The observed anti-inflammatory activities of both extracts are consistent with previous reports describing the presence of phenolic compounds and other bioactive constituents in members of the family Zingiberaceae, which have been associated with the inhibition of protein denaturation and other inflammation-related pathways [25–27]. However, the present study primarily aimed to demonstrate the applicability of the developed analytical method rather than to comprehensively investigate the pharmacological properties of the tested plant extracts. Additionally, a direct comparison of the plant extracts IC_{50} values between the developed microplate-based assay and the conventional tube-based method was not performed in this work. While the analytical performance and equivalence of the microplate protocol were fully validated using the reference standard (diclofenac sodium), the lack of comparative data for complex natural extracts represents a limitation of the current study. Future research should focus on comparative testing of a wider range of diverse plant extracts using both platforms to further confirm the robust applicability and analytical agreement of the developed method for natural product screening.

Table 3
Anti-inflammatory activity of ethanolic extracts of *Zingiber montanum* and *Etlingera pavieana* determined by the protein denaturation assay.

Plant extracts	Concentration (µg/mL)	Inhibition (%)±SD	IC_{50} (µg/mL)
Zingiber montanum	5	22.93 ± 4.66	13.31 ± 3.00[a]
	10	45.09 ± 3.73	
	20	60.78 ± 6.29	
Etlingera pavieana	5	23.41 ± 4.94	14.31 ± 0.79[a]
	10	41.74 ± 6.44	
	20	64.53 ± 5.56	

*Data are presented as mean±SD of three independent experiments (n=3), with each experiment analyzed in triplicate.Values in the IC_{50} column followed by the same superscript letter (a) are not significantly different (p>0.05).

4. Conclusions

A microplate-based BSA protein denaturation assay was successfully developed and optimized for the preliminary screening of anti-inflammatory activity. Compared with the conventional tube-based assay, the proposed method reduced the total reaction volume and sample consumption and shortened the overall analysis time while maintaining comparable experimental outcomes. The developed assay was successfully applied to evaluate the anti-inflammatory activities of ethanolic extracts of *Zingiber montanum* and *Etlingera pavieana,* demonstrating its applicability for the rapid screening of natural products. In addition to improving laboratory efficiency, the proposed method offers a simple, cost-effective, reproducible, and high-throughput analytical platform suitable for routine laboratory applications. The developed microplate assay therefore represents a practical alternative to the conventional protein denaturation method

and may facilitate future studies involving the preliminary evaluation of anti-inflammatory compounds from natural and synthetic sources.

Acknowledgements

The authors would like to express their sincere gratitude to the Faculty of Science and Technology, Rambhai Barni Rajabhat University, Thailand, for providing laboratory facilities and research support. The authors also thank all colleagues and staff who assisted with the experimental work and provided valuable technical support throughout this study.

References

[1] Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature, 454*(7203), 428–435. https://doi.org/10.1038/nature07201.

[2] Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2018). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget, 9*(6), 7204–7218. https://doi.org/10.18632/oncotarget.23208.

[3] Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., Ferrucci, L., Gilroy, D. W., Fasano, A., Miller, G. W., Miller, A. H., Mantovani, A., Weyand, C. M., Barzilai, N., Goronzy, J. J., Rando, T. A., Effros, R. B., Lucia, A., Kleinstreuer, N., & Slavich, G. M. (2019). Chronic inflammation in the etiology of disease across the life span. *Nature Medicine, 25*(12), 1822–1832. https://doi.org/10.1038/s41591-019-0675-0.

[4] Vane, J. R., & Botting, R. (1987). Inflammation and the mechanism of action of anti-inflammatory drugs. *The FASEB Journal, 1*(2), 89–96. https://doi.org/10.1096/fasebj.1.2.3111928.

[5] Atanasov, A. G., Zotchev, S. B., Dirsch, V. M., & International Natural Product Sciences Taskforce. (2021). Natural products in drug discovery: Advances and opportunities. *Nature Reviews Drug Discovery, 20*(3), 200–216. https://doi.org/10.1038/s41573-020-00114-z.

[6] Gunathilake, K. D. P. P., Ranaweera, K. K. D. S., & Rupasinghe, H. P. V. (2018). In vitro anti-inflammatory properties of selected green leafy vegetables. *Biomedicines, 6*(4), Article 107. https://doi.org/10.3390/biomedicines6040107.

[7] Juvekar, A., Sakat, S., Wankhede, S., Juvekar, M., & Gambhire, M. (2009). Evaluation of antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculata*. *Planta Medica, 75*(9), Article PJ178. https://doi.org/10.1055/s-0029-1234983.

[8] Williams, L. A. D., O'Connar, A., Latore, L., Dennis, O., Ringer, S., Whittaker, J. A., Conrad, J., Vogler, B., Rosner, H., & Kraus, W. (2008). The in vitro anti-denaturation effects induced by natural products and non-steroidal compounds in heat-treated (immunogenic) bovine serum albumin as a screening assay for the detection of anti-inflammatory compounds, without the use of animals, in the early stages of the drug discovery process. *West Indian Medical Journal, 57*(4), 327–331.

[9] Mizushima, Y., & Kobayashi, M. (1968). Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins. *Journal of Pharmacy and Pharmacology, 20*(3), 169–173. https://doi.org/10.1111/j.2042-7158.1968.tb09718.x.

[10] Peiris, D. S. H. S., Fernando, D. T. K., Senadeera, S. P. N. N., & Ranaweera, C. B. (2025). Assessment of in vitro anti-inflammatory activity: A comprehensive review of methods, advantages, and limitations. *Asian Journal of Research in Biochemistry, 15*(2), 37–52. https://doi.org/10.9734/ajrb/2025/v15i2365.

[11] Chandra, S., Chatterjee, P., Dey, P., & Bhattacharya, S. (2012). Evaluation of in vitro anti-inflammatory activity of coffee against the denaturation of protein. *Asian Pacific Journal of Tropical Biomedicine, 2*(1, Suppl.), S178–S180. https://doi.org/10.1016/S2221-1691(12)60154-3.

[12] Precupas, A., & Popa, V. T. (2024). Impact of sinapic acid on bovine serum albumin thermal stability. *International Journal of Molecular Sciences, 25*(2), Article 936. https://doi.org/10.3390/ijms25020936.

[13] Sakouhi, S., Ben Younes, S., Arrari, F., Nahdi, A., Bouajila, J., & Mhamdi, A. (2025). Integrated in vitro and in silico characterization of 5-hydroxyferulic acid: Antioxidant, anti-inflammatory, anti-hemolytic, and cytotoxic potential. *Chemistry & Biodiversity, 22*(12), Article e01431. https://doi.org/10.1002/cbdv.202501431.

[14] Nirmala, A. R., Permatasari, L., Muliasari, H., & Deccati, R. F. (2023). Review: Analysis of optimal conditions of bovine serum albumin (BSA) protein denaturation inhibition method in anti-inflammatory activity testing of various plant leaf extracts. *Journal of Agritechnology and Food Processing, 3*(2), 101–113. https://doi.org/10.31764/jafp.v3i2.20953.

[15] Williams, L. A. D. (2009). Further insight into the bovine serum albumin assay (the in vitro anti-inflammatory assay). *West Indian Medical Journal, 58*(2), 181–182.

[16] Inglese, J., Johnson, R. L., Simeonov, A., Xia, M., Zheng, W., Austin, C. P., & Auld, D. S. (2007). High-throughput screening assays for the identification of chemical probes. *Nature Chemical Biology, 3*(8), 466–479. https://doi.org/10.1038/nchembio.2007.17.

[17] Macarron, R., Banks, M. N., Bojanic, D., Burns, D. J., Cirovic, D. A., Garyantes, T., Green, D. V. S., Hertzberg, R. P., Janzen, W. P., Paslay, J. W., Schopfer, U., & Sittampalam, G. S. (2011). Impact of high-throughput screening in biomedical research. *Nature Reviews Drug Discovery, 10*(3), 188–195. https://doi.org/10.1038/nrd3368.

[18] Janzen, W. P. (2014). Screening technologies for small molecule discovery: The state of the art. *Chemistry & Biology, 21*(9), 1162–1170. https://doi.org/10.1016/j.chembiol.2014.07.015.

[19] Auld, D. S., Coassin, P. A., Coussens, N. P., Hensley, P., Klumpp-Thomas, C., Michael, S., Sittampalam, G. S., Trask, O. J., Wagner, B. K., Weidner, J. R., Wildey, M. J., & Dahlin, J. L. (2020). Microplate selection and recommended practices in high-throughput screening and quantitative biology. In S. Markossian, A. Grossman, K. Brimacombe, M. Arkin, D. Auld, C. Austin, J. Baell, T. D. Y. Chung, N. P. Coussens, J. L. Dahlin, V. Devanarayan, T. L. Foley, M. Glicksman, M. D. Hall, J. V. Haas, S. R. J. Hoare, J. Inglese, P. W. Iversen, S. D. Kahl, S. C. Kales, M. Lal-Nag, Z. Li, J. McGee, O. McManus, T. Riss, O. J. Trask, J. R. Weidner, M. J. Wildey, M. Xia, & X. Xu (Eds.), *Assay guidance manual*. Eli Lilly & Company and the National Center for Advancing Translational Sciences. https://www.ncbi.nlm.nih.gov/books/NBK558077/.

[20] Ravindranath, K. J., & Srinivasan, H. (2025). Deciphering the anti-neoplastic potential of *Allium ascalonicum* in averting the proliferation and epithelial–mesenchymal transition of triple-negative breast cancer through virtual docking and in vitro approaches. *BMC Cancer, 25*, Article 414. https://doi.org/10.1186/s12885-025-13796-8.

[21] Nirmal, N. P., & Panichayupakaranant, P. (2015). Antioxidant, antibacterial, and anti-inflammatory activities of standardized brazilin-rich *Caesalpinia sappan* extract. *Pharmaceutical Biology, 53*(9), 1339–1343. https://doi.org/10.3109/13880209.2014.982295.

[22] Mustafa, A., Indiran, M. A., Ramalingam, K., & Shanmugam, R. (2023). Evaluation of the anti-inflammatory, antimicrobial, antioxidant, and cytotoxic effects of chitosan thiocolchicoside–lauric acid nanogel. *Cureus, 15*(9), Article e46003. https://doi.org/10.7759/cureus.46003.

[23] Nandita, R., Jeevitha, M., Kaarthikeyan, G., & Raj, K. (2024). Anti-inflammatory activity and free radical scavenging activity of *Tridax procumbens* leaves-based chitosan gel. *Journal of Pharmacy and Bioallied Sciences, 16* (Suppl. 4), S4081–S4084. https://doi.org/10.4103/jpbs.jpbs_1439_24.

[24] Rahim, A., Mim, F., Reza, M. S., Dey, S. K., Shoagh, M. S. R., Islam, M. B., & Nasiruddin, M. (2025). Unveiling the phytochemical profile, antioxidant capacity, cytotoxicity, and in vitro anti-inflammatory effect of three leafy green vegetables: *Digera muricata*, *Trianthema portulacastrum*, and *Alternanthera philoxeroides*. *Food Chemistry: X, 29*, Article 102620. https://doi.org/10.1016/j.fochx.2025.102620.

[25] Ozaki, Y., Kawahara, N., & Harada, M. (1991). Anti-inflammatory effect of *Zingiber cassumunar* Roxb. and its active principles. *Chemical & Pharmaceutical Bulletin, 39*(9), 2353–2356. https://doi.org/10.1248/cpb.39.2353.

[26] Jeenapongsa, R., Yoovathaworn, K., Sriwatanakul, K. M., Pongprayoon, U., & Sriwatanakul, K. (2003). Anti-inflammatory activity of (E)-1-(3,4-dimethoxyphenyl)butadiene from *Zingiber cassumunar* Roxb. *Journal of Ethnopharmacology, 87*(2–3), 143–148. https://doi.org/10.1016/S0378-8741(03)00098-9.

[27] Srisook, E., Palachot, M., Mankhong, S., & Srisook, K. (2017). Anti-inflammatory effect of *Etlingera pavieana* (Pierre ex Gagnep.) R.M.Sm. rhizomal extract and its phenolic compounds in lipopolysaccharide-stimulated macrophages. *Pharmacognosy Magazine, 13*(Suppl. 2), S230–S235. https://doi.org/10.4103/pm.pm_558_16.