Anti-Inflammatory Effects of *Cassia alata* L. in Human Keratinocytes

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Abstract

This study aimed to evaluate the antioxidant activity, cytotoxicity, and anti-inflammatory effects of *Cassia alata* L. leaf extract in human keratinocyte (HaCaT). Cytotoxicity determined by the resazurin assay revealed that the extract exhibited low toxicity at concentrations below 100 µg/mL, with cell viability remaining above 80–90%. Antioxidant evaluation using the DPPH assay demonstrated moderate radical scavenging activity, with an IC_{50} value of 77.95 µg/mL. The anti-inflammatory effects assessed through the nitric oxide assay, expressed as normalized % of LPS, indicated that the 12.5 µg/mL extract significantly inhibited LPS-induced nitric oxide production, reducing nitrite levels to approximately 0–5% of LPS, comparable to dexamethasone (100 nM). The 25 µg/mL extract also significantly reduced NO production, although to a lesser extent. These findings suggest that *C. alata* leaf extract possesses promising anti-inflammatory potential with acceptable cellular safety profiles, supporting its application in anti-inflammatory herbal preparations or skin-related cosmeceutical formulations.

Keywords: *Cassia alata* L., keratinocytes, inflammation, antioxidants, nitric oxide, HaCaT cells

1. Introduction

Inflammatory skin disorders such as atopic dermatitis and psoriasis are chronic and recurrent conditions characterized by persistent epidermal inflammation mediated by dysregulated cytokine release and excessive production of reactive oxygen species (ROS) from keratinocytes and immune cells (Kim & Lee, 2016; Nestle et al., 2009). Keratinocytes, which constitute the majority of the epidermis, serve as both a physical barrier and an active immune regulator; however, under pathological stimuli, they generate ROS and pro-inflammatory mediators that exacerbate cutaneous inflammation and impair barrier function (Madison, 2003; Proksch et al., 2008). As oxidative stress and inflammatory signaling are key contributors to disease progression, strategies aimed at modulating these pathways at the cellular level are essential

for developing safer and more effective treatments for chronic inflammatory skin diseases (Leung et al., 2004).

Cassia alata L., a traditional medicinal plant widely used in Southeast Asia, has been reported to possess multiple biological activities relevant to skin health, including antioxidant and anti-inflammatory properties (Agampodi & Collet, 2022; Subramanian et al., 2015). Bioactive constituents such as flavonoids and anthraquinones have been shown to reduce oxidative stress and attenuate inflammatory responses, particularly by inhibiting nitric oxide (NO) production in immune cells (Kandiah et al., 2025; Pongnimitprasert et al., 2018). These findings suggest that *C. alata* may serve as a promising natural therapeutic candidate for managing skin inflammation and restoring epidermal homeostasis.

Despite these promising observations, evidence regarding the biological effects of *C. alata* leaf extract on human keratinocytes remains limited. In particular, data are scarce on its antioxidant capacity, cytotoxicity, and inhibitory effects on NO production in HaCaT cells, a well-established human keratinocyte model used to study skin inflammation. Addressing these gaps is critical for substantiating the potential dermatological applications of *C. alata* and supporting its development for safe and effective herbal-based therapies.

Therefore, this study aimed to evaluate the antioxidant activity, cytotoxic effects, and NO-inhibitory potential of *Cassia alata* leaf extract in human keratinocyte (HaCaT) cells. The findings provide important insights into the potential use of *C. alata* as a natural anti-inflammatory and skin-protective agent and contribute to the advancement of herbal-based innovations for managing inflammatory skin disorders.

1.1 Objectives

1. To evaluate the antioxidant activity and cytotoxicity of *Cassia alata* L. leaf extract in human keratinocyte (HaCaT) cells.
2. To investigate the inhibitory effect of *Cassia alata* L. leaf extract on nitric oxide (NO) production in human keratinocyte (HaCaT) cells.

2. Materials and methods

Chemicals

HyClone DMEM/LOW medium supplemented with L-glutamine and sodium pyruvate, fetal bovine serum (FBS; HyClone, SV30160.03), penicillin–streptomycin solution (Corning, SH30034.02), phosphate-buffered saline (PBS; HyClone, SH30258.02), and 0.25% EDTA–trypsin (Capricorn, TRY-1810) were used for HaCaT cell culture and maintenance. Resazurin sodium salt (Sigma-Aldrich, SIA-R7017) was used for cytotoxicity assessment. DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich, Germany) and butylated hydroxytoluene (BHT) were used for antioxidant analysis. Lipopolysaccharide (LPS) was used to induce nitric oxide production, while sodium nitrite ($NaNO_2$; AR grade, KemAus), sulfanilamide, phosphoric acid, and N-(1-naphthyl)ethylenediamine dihydrochloride were used for NO quantification using the

Griess reaction. Dimethyl sulfoxide (DMSO; Biochemical, PC0906) and absolute ethanol (AR grade) were used for reagent preparation. All solutions were prepared using deionized water.

Cell culture

HaCaT cells (human immortalized keratinocyte cell line) were obtained from CLS Cell Lines Service GmbH (Cytion, catalog number 300493). The cells were cultured in HyClone DMEM/LOW medium supplemented with 10% fetal bovine serum (FBS; HyClone, SV30160.03), 1% penicillin–streptomycin solution (Corning, SH30034.02), and L-glutamine. Cells were maintained at 37 °C in a humidified incubator with 5% CO_2. HaCaT cells were subcultured using 0.25% EDTA–trypsin (Capricorn, TRY-1810) when reaching approximately 70–80% confluence prior to experiments.

Herbal Extraction

Fresh leaves of *Cassia alata* L. were dried in a hot-air oven at 50 °C for 6 hours, and the moisture content was adjusted to not more than 5%. The dried plant material (500 g) was then macerated in 95% ethanol (5 L) for 3 days at room temperature. The extract was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated under reduced pressure using a vacuum rotary evaporator to remove the solvent. The concentrated extract was further dried in a hot-air oven until all residual solvent had completely evaporated. The dried crude extract was stored in an airtight container at 4 °C until further use (Sudjaroen, Y., & Thongmuang, P. 2022).

DPPH Radical Scavenging Assay

The antioxidant activity of the extract was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. A 0.1 mM DPPH solution was freshly prepared in absolute ethanol, protected from light, and used immediately. The crude extract and the standard antioxidant butylated hydroxytoluene (BHT) were dissolved in absolute ethanol to obtain stock concentrations of 12.5, 50, 100, 200, and 400 µg/mL. The assay was performed by mixing equal volumes (1:1, v/v) of each sample solution with the DPPH solution, resulting in final test concentrations of 6.25, 12.5, 50, 100, and 200 µg/mL. Absolute ethanol served as the blank control.

The reaction mixtures were incubated in the dark for 30 minutes at room temperature and subsequently measured at 520 nm using a microplate reader. All experiments were performed in triplicate. The percentage of DPPH radical scavenging activity (%RSA) was calculated using the following equation:

$$\% RSA = [\frac{^{1} - \mathrm{Abs}_{\mathrm{sample}}}{\mathrm{Abs}_{\mathrm{control}}}]\, x\, 100$$

where A_{control} is the absorbance of the DPPH solution without sample, and A_{sample} is the absorbance of the sample–DPPH mixture. The half-maximal effective concentration (EC_{50}) was obtained from the dose–response curve using linear regression analysis.

Resazurin Assay for Cytotoxicity

The cytotoxicity of the *Cassia alata* leaf extract was assessed using the resazurin reduction assay (Lee et al. 2018). HaCaT cells were seeded into 96-well plates at a density of 20,000 cells per well in 200 µL of complete growth medium (CGM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 hours until reaching approximately 60% confluence. The culture medium was then replaced with medium containing the extract at final concentrations of 50, 100, 250, 500, and 1,000 µg/mL (200 µL per well). Cells treated with 0.1% Triton X-100 served as the negative control for complete cell death.

Media were refreshed every 48 hours, and cell viability was measured after 24 hours of treatment. The culture medium was removed, and cells were washed twice with 100 µL of phosphate-buffered saline (PBS). Subsequently, 100 µL of fresh medium containing 10% resazurin solution was added to each well. Plates were incubated at 37 °C in 5% CO_2 for 4 hours in the dark, after which absorbance was measured at 570 nm and 600 nm using a microplate reader.

Cell viability (%) was calculated using the following equation:

$$\%\ Cell\ viability = \left(\frac{OD_{sample} - blank\ absorbance}{Mean\ OD_{control} - blank\ absorbance}\right) \times 100$$

Nitric Oxide Determination Using the Griess Assay

Nitric oxide (NO) production was quantified by measuring nitrite accumulation in the culture supernatant using the Griess reaction, following the method described by Kim et al. (2023) with slight modifications. HaCaT cells were seeded into 96-well plates at a density of 1×10^4 cells per well in 200 µL of complete growth medium (CGM) and incubated at 37 °C in a humidified incubator containing 5% CO_2 for 48 hours. Cells were then pretreated with *Cassia alata* extract (200 µL/well) for 4 hours, followed by stimulation with lipopolysaccharide (LPS) at a final concentration of 1 µg/mL. After 20 hours of LPS stimulation, the culture supernatant was collected for nitrite analysis.

For NO quantification, equal volumes of cell culture supernatant were mixed with freshly prepared Griess reagent, consisting of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (NED). The mixture was incubated at room temperature for 10 minutes, protected from light. Absorbance was then measured at 540 nm using a microplate reader. Nitrite concentrations were determined using a sodium nitrite ($NaNO_2$) standard curve and expressed relative to the LPS control.

Statistical Analysis

All experiments were performed in three independent replicates, and data are presented as mean ± standard error of the mean (SEM). Statistical analyses were conducted using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA). For comparisons among multiple treatment groups, one-way analysis of variance (one-way ANOVA) was performed after

verifying normality and homogeneity of variance using the Shapiro–Wilk test and Brown–Forsythe test, respectively. When significant differences were detected, Tukey's multiple comparison test was used as the post hoc test to determine pairwise differences among groups. A confidence level of 95% was used for all tests, and statistical significance was defined as $p < 0.05$.

3. Results

Antioxidant activity

The antioxidant activity of *Cassia alata* leaf extract was evaluated using the DPPH radical scavenging assay and compared with the standard antioxidant BHT. Both the extract and BHT exhibited dose-dependent increases in radical scavenging activity. However, BHT showed significantly higher %RSA at all tested concentrations. At 50 µg/mL, BHT demonstrated 67.64% scavenging activity, while the extract showed 32.03%. The IC_{50} values were 14.41 µg/mL for BHT and 77.95 µg/mL for the extract, indicating that *Cassia alata* possesses moderate antioxidant capacity relative to the chemical standard.

Table 1 DPPH radical scavenging activity (%RSA) and IC_{50} values of *Cassia alata* leaf extract compared with BHT

Concentration (µg/ml)	**BHT**		***Cassia alata* L.**	
	%RSA	**IC_{50} (µg/ml)**	**%RSA**	**IC_{50} (µg/ml)**
3.125	15.78	14.41	3.48	77.95
6.25	24.60		6.29	
12.5	39.42		11.05	
25	53.17		20.05	
50	67.64		32.03	

Cytotoxicity

The cytotoxicity of *Cassia alata* leaf extract on HaCaT keratinocytes was assessed using the resazurin reduction assay. The extract exhibited a dose-dependent effect on cell viability. At low concentrations (12.5–50 µg/mL), cell viability remained above 90%, indicating minimal cytotoxicity. Moderate cytotoxicity was observed at 100–250 µg/mL, with viability decreasing to approximately 80% and 60–70%, respectively. Higher concentrations (500–1000 µg/mL) resulted in a marked reduction in viability, with fewer than 15% of cells remaining viable at 1000 µg/mL. The results confirm that concentrations ≤100 µg/mL are safe for further biological assays.

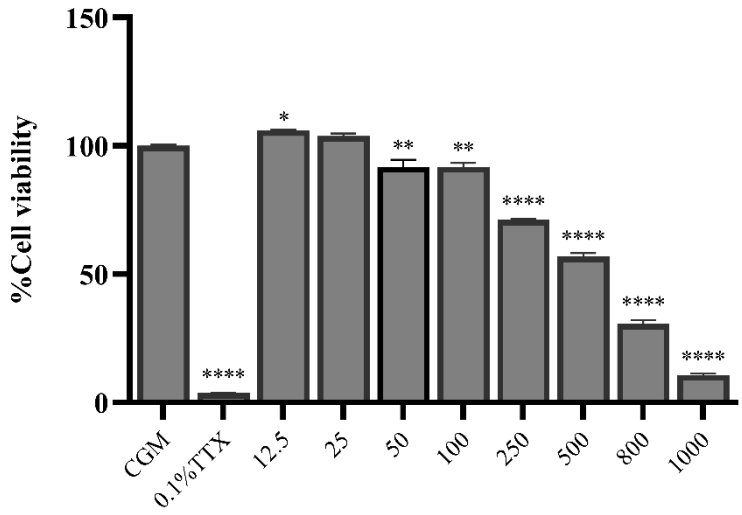

Figure 1 Cytotoxic effects of *Cassia alata* leaf extract on HaCaT cells determined by the resazurin assay. Cell viability is expressed as percentage of the untreated control (CGM). Data represent mean ± SEM (n = 3). Statistical significance relative to control is indicated as $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$.

Anti-inflammatory activity

The inhibitory effect of *Cassia alata* leaf extract on nitric oxide production was evaluated in LPS-stimulated HaCaT cells using the Griess assay, and results were expressed as % of the LPS control. As shown in Figure X, LPS stimulation markedly increased nitrite accumulation, which was defined as 100% inflammatory response. Treatment with dexamethasone (100 nM) significantly suppressed NO production to approximately 0–3% of LPS, confirming its role as the positive anti-inflammatory control.

Pretreatment with *C. alata* extract at 12.5 μg/mL significantly reduced NO production to approximately 0– 5% of LPS, demonstrating strong inhibitory activity comparable to dexamethasone. The 25 μg/mL concentration also significantly decreased NO levels, reducing nitrite to approximately 35–40% of LPS, although its effect was weaker than the 12.5 μg/mL treatment.

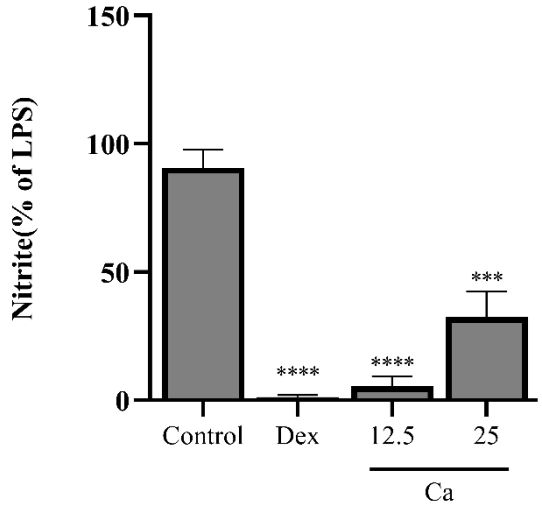

Figure 2 Inhibitory effects of *Cassia alata* leaf extract on LPS-induced nitric oxide production in HaCaT cells. Nitrite levels were quantified using the Griess assay and expressed as percentage of LPS control (% of LPS). Data represent mean ± SEM (n = 3). Statistical significance relative to LPS control is indicated as ****$p < 0.0001$ and ***$p < 0.001$.

Overall, these findings indicate that *Cassia alata* leaf extract effectively inhibits LPS-induced NO production in HaCaT cells, with the strongest effect observed at 12.5 µg/mL. This suggests that the extract may modulate inflammatory responses, potentially through suppression of iNOS-mediated nitric oxide synthesis.

4. Conclusion

This study demonstrated that the leaf extract of *Cassia alata* L. possesses notable biological activities relevant to skin health, including low cytotoxicity, moderate antioxidant capacity, and strong anti-inflammatory potential in HaCaT keratinocytes. Cytotoxicity testing using the resazurin assay showed that the extract was safe at concentrations below 100 µg/mL, maintaining more than 80–90% cell viability, while higher concentrations (>250 µg/mL) exhibited significant dose-dependent cytotoxic effects. Antioxidant evaluation with the DPPH assay revealed moderate radical scavenging activity, with an IC_{50} of 77.95 µg/mL, indicating a lower potency compared with the chemical standard BHT but confirming the extract's ability to reduce free radicals.

Importantly, the extract demonstrated strong anti-inflammatory activity, as evidenced by its ability to suppress LPS-induced nitric oxide production. The 12.5 µg/mL concentration markedly reduced nitrite levels to near baseline, comparable to dexamethasone (100 nM), while the 25 µg/mL concentration also significantly inhibited NO production, albeit to a lesser extent. These findings suggest that *C. alata* extract effectively modulates inflammatory responses, potentially through the suppression of iNOS-related NO synthesis and attenuation of NF-κB–associated signaling pathways.

Overall, the results support the potential of *Cassia alata* as a natural source of bioactive compounds with anti-inflammatory and antioxidant activities. Future studies should focus on identifying the specific active constituents, investigating molecular mechanisms such as iNOS, COX-2, and NF-κB expression, and evaluating intracellular oxidative stress using complementary assays. Additional safety assessments in other skin cell types, irritation testing, and in vivo studies will be essential to validate its suitability for cosmeceutical or herbal therapeutic applications. At appropriate low concentrations, the extract shows promising potential for use in formulations targeting skin inflammation and oxidative stress.

Acknowledgment

This work was supported by (I) Chutikarn Sriviboon, President of Suan Sunandha Rajabhat University, Thailand. and (II) Somdech Rungsrisawat, an Associate Professor and the Dean of College of Allied Health Sciences, Suan Sunandha Rajabhat University, Thailand.

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