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# Comparative Antiviral Activity of Clinacanthus nutans Extracts Obtained by Soxhlet Extraction and Fermentation against HSV-1

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## ABSTRACT

Clinacanthus nutans is a medicinal plant traditionally used for viral infections; however, the influence of extraction methods on its antiviral efficacy remains poorly understood. This study comparatively evaluated the anti-herpes simplex virus type-1 (HSV-1) activity of C. nutans leaf extracts obtained by Soxhlet extraction and fermentation. Antiviral activity was assessed in HSV-1-infected Vero cells using a GFP-based assay across multiple concentrations (0.206–50 µg/mL), together with cytotoxicity evaluation. Both extracts exhibited non-cytotoxic profiles at all tested concentrations. The Soxhlet extract showed moderate antiviral inhibition (18.45–27.15%), with maximal activity at 1.85 µg/mL (27.15%), while the fermented extract demonstrated consistently higher inhibition (22.31–32.08%), reaching a maximum of 32.08% at 50 µg/mL. Although neither extract achieved the ≥50% threshold for classification as an active antiviral agent, fermentation clearly enhanced antiviral performance compared with Soxhlet extraction. These findings indicate that extraction strategy significantly influences the biological activity of C. nutans and highlight fermentation as a promising bioprocessing approach to enhance its antiviral potential, providing a foundation for further phytochemical characterization and bioactivity-guided compound isolation.

***Keywords:*** *Clinacanthus nutans; Soxhlet extraction; fermentation; HSV-1; antiviral activity*

## 1. Introduction

Herpes simplex virus type-1 (HSV-1) remains a globally prevalent viral pathogen responsible for a wide range of clinical manifestations, including orofacial lesions, keratitis, and severe complications in immunocompromised individuals. Although antiviral agents such as acyclovir are widely used in clinical practice, increasing reports of drug resistance, limited long-term efficacy, and adverse effects highlight the urgent need for alternative antiviral agents derived from safer and more sustainable sources [16,24,26].

Natural products and medicinal plants have therefore gained growing scientific attention as promising reservoirs of novel antiviral compounds [16,18,24,26,27,28,29,30].

*Clinacanthus nutans* (Burm.f.) Lindau is a medicinal plant traditionally used in Southeast Asia for the treatment of viral infections, inflammatory disorders, and skin diseases [1,5,20]. Ethnopharmacological applications and increasing experimental evidence [7] suggest that *C. nutans* possesses broad biological activities, including antioxidant, anti-inflammatory, and antiviral effects [1,6,9,10,20]. Phytochemical investigations have demonstrated that the plant contains multiple bioactive constituents, particularly flavonoids, phenolic compounds, and glycosides, which are known to exhibit antiviral potential against various viral pathogens [1,6,18,20,27,28]. Despite these promising properties, the translation of traditional usage into scientifically validated antiviral applications remains limited, particularly with respect to standardized extraction strategies [1,5,6,20].

Extraction methodology plays a critical role in determining the chemical composition, biological activity, and reproducibility of plant-derived products [1,6,20]. Conventional techniques such as Soxhlet extraction are widely used for their efficiency, reproducibility, and ability to exhaustively extract bioactive compounds under controlled conditions. In contrast, fermentation represents a biological processing approach that can modify phytochemical profiles through microbial biotransformation, potentially enhancing biological activity by converting inactive precursors into more bioavailable or bioactive forms.

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However, comparative evaluations of these two fundamentally different extraction strategies in the context of antiviral activity remain scarce [1,6,20].

Current studies on *C. nutans* have primarily focused on either phytochemical characterization or biological screening independently, with limited integration between extraction methodology and antiviral efficacy [2,3,4]. In particular, systematic comparisons between physicochemical extraction methods and bioprocessing approaches, such as fermentation, in relation to HSV-1 inhibition are lacking. Understanding how extraction strategies influence antiviral potential is essential for developing standardized, reproducible, and biologically effective herbal-derived antiviral formulations [1,6,9,10,20].

Therefore, this study aimed to comparatively evaluate the antiviral activity of *Clinacanthus nutans* leaf extracts obtained by Soxhlet extraction and fermentation against HSV-1 using a cell-based antiviral model. By assessing antiviral inhibition across multiple concentrations and confirming cellular safety profiles, this work seeks to establish a scientific foundation for extraction method–guided optimization of *C. nutans* as a potential source of antiviral bioactive compounds and to support future phytochemical characterization and bioactivity-guided compound isolation strategies.

## 2. Materials and Methods

### 2.1 Plant Material

Fresh leaves of Clinacanthus nutans (Burm.f.) Lindau were collected from [location], Thailand. The plant material was authenticated by a botanist [11,12], and a voucher specimen was deposited at [herbarium/institution, if available]. Leaves were washed with distilled water, air-dried at room temperature, and subsequently oven-dried at controlled temperature until constant weight was achieved. The dried leaves were ground into fine powder and stored in airtight containers at room temperature until extraction [16].

### 2.2 Soxhlet Extraction

Powdered C. nutans leaves were subjected to Soxhlet extraction using ethanol as the extraction solvent [13,14,15]. Briefly, the plant material was placed in a cellulose extraction thimble and extracted using a Soxhlet apparatus for continuous reflux cycles. The extraction process was conducted under controlled heating until exhaustive extraction was achieved. The resulting extract solution was filtered and concentrated under reduced pressure using a rotary evaporator. The crude extract was further dried in a controlled environment for 18 h to remove residual solvent. The dried extract was designated as S0144 (Soxhlet extract) and stored at 4 °C until use.

### 2.3 Fermentation Extraction

Fermentation was performed using powdered C. nutans leaves under controlled conditions. The plant material was suspended in sterile medium and subjected to microbial fermentation under appropriate temperature and time conditions. After completion of fermentation, the mixture was filtered to remove solid residues, and the liquid extract was concentrated under reduced pressure. The concentrated extract was dried and stored at 4 °C. This extract was designated as S0145 (fermented extract) for subsequent biological evaluation.

### 2.4 Preparation of Extract Solutions

Both dried extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions and further diluted with cell culture medium to achieve final working concentrations of 0.206, 0.617, 1.85, 5.56, 16.67, and 50 µg/mL using a 1:3 serial dilution strategy. The final concentration of DMSO in all test solutions was maintained at 0.5% (v/v). A 0.5% DMSO solution was used as the negative control.

### 2.5 Cell Culture

Vero cells (kidney epithelial cells derived from African green monkey [25,26,28], Chlorocebus sabaeus) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and maintained at 37 °C in a humidified incubator with 5% $Co_2$.

### 2.6 Virus Preparation

Herpes simplex virus type-1 (HSV-1) was propagated in Vero cells and harvested according to standard virological procedures. Viral stocks were stored at −80 °C until use. Viral titers were determined prior to antiviral assays [11,15].

### 2.7 Antiviral Assay

Antiviral activity was evaluated using a GFP-based antiviral assay in HSV-1-infected Vero cells. Cells were seeded into 96-well plates and incubated overnight to allow cell attachment. HSV-1 was then added to the wells, followed by treatment with the test extracts at the indicated concentrations. After incubation for 18 h, viral replication was quantified based on GFP fluorescence intensity. The percentage of viral inhibition was calculated relative to virus control wells. Acyclovir was used as the positive control, while 0.5% DMSO served as the negative control.

### 2.8 Cytotoxicity Assay

Cytotoxicity of both extracts was assessed in Vero cells using the same concentration range as in the antiviral assay. Cell viability was determined using a GFP-based viability evaluation system. The percentage of cell viability was calculated relative to untreated control cells. Extracts showing no significant reduction in cell viability were considered non-cytotoxic.

### 2.9 Data Analysis

All experiments were performed in triplicate, and data were expressed as mean values [23]. Antiviral activity was presented as percentage inhibition of HSV-1 replication. Cytotoxicity was expressed as percentage cell viability relative to control. Data analysis and graphical visualization were conducted using standard statistical software [8].

### 2.10 Ethical and Biosafety Compliance

All experiments involving HSV-1 and cell cultures were conducted in accordance with institutional biosafety guidelines and laboratory safety regulations.

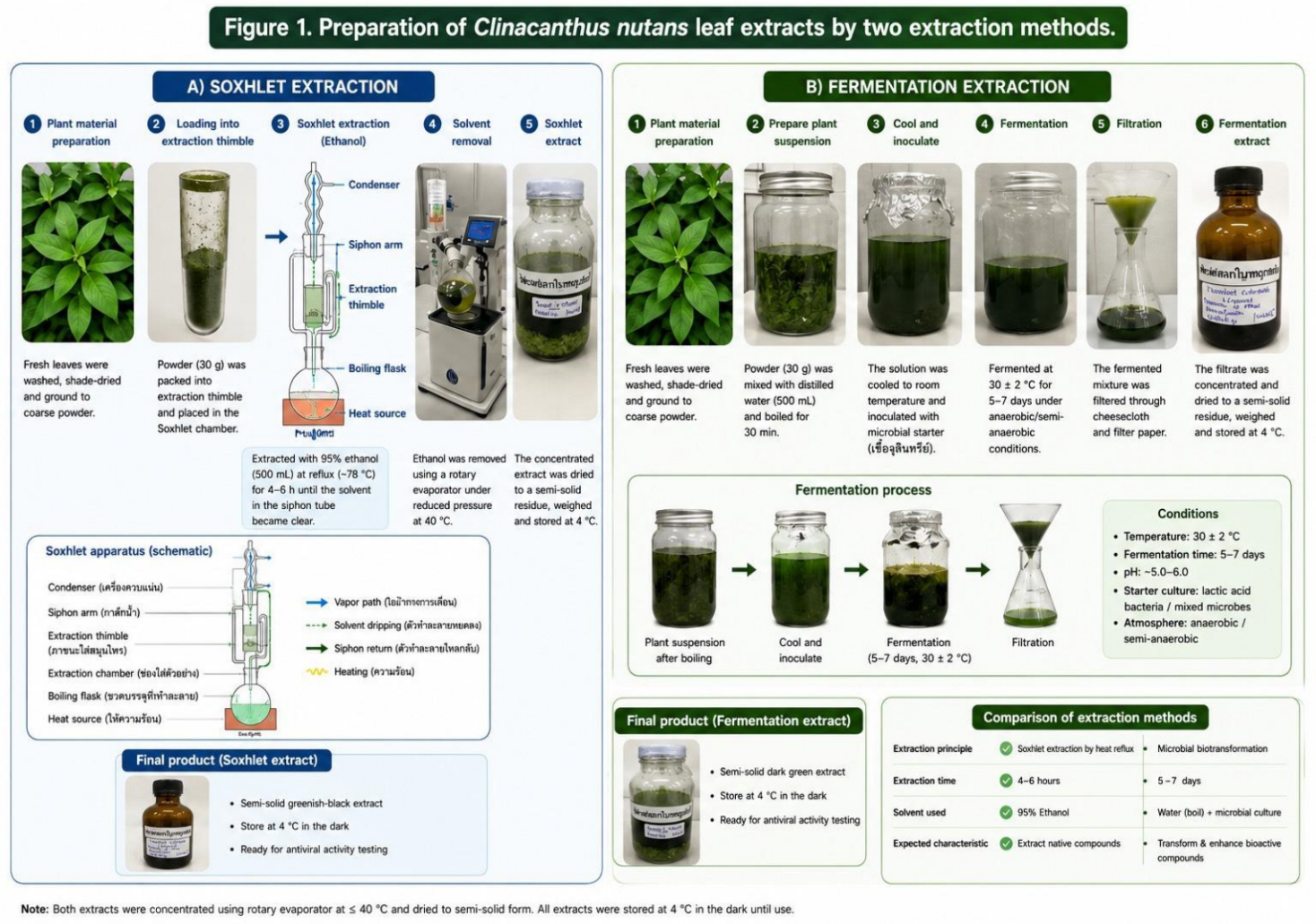


*Figure 1. Preparation of Clinacanthus nutans leaf extracts*

## 3. Results

### 3.1 Cytotoxicity Evaluation

Both Soxhlet and fermented extracts of Clinacanthus nutans were first evaluated for cytotoxic effects in Vero cells across the tested concentration range (0.206–50 µg/mL). Neither extract induced significant cytotoxicity at any concentration, as cell viability remained comparable to the negative control (0.5% DMSO). These findings indicate that both extracts exhibited a favorable cellular safety profile and were suitable for subsequent antiviral evaluation without confounding effects from extract-induced cytotoxicity.

*Table 1: Description of Clinacanthus nutans Extracts Used in the Study*

| Sample Code | Plant Part | Extraction Method | Solvent/Process | Processing Type | Purpose in Study |
|---|---|---|---|---|---|
| S0144 | Leaves | Soxhlet extraction | Ethanol | Physicochemical extraction | Antiviral evaluation |
| S0145 | Leaves | Fermentation | Microbial fermentation process | Bioprocessing extraction | Antiviral evaluation |

**Caption:**

*Description of Soxhlet and fermented extracts of Clinacanthus nutans prepared for comparative antiviral evaluation.*

### 3.2 Antiviral Activity of Soxhlet Extract (S0144)

The Soxhlet extract (S0144) demonstrated measurable but moderate inhibitory activity against HSV-1 across all tested concentrations. The percentage inhibition ranged from 18.45% to 27.15%. The highest antiviral activity was observed at 1.85 µg/mL, where the extract achieved 27.15% inhibition of viral replication. No clear dose-dependent trend was observed, as inhibition values remained within a relatively narrow range across concentrations (0.206–50 µg/mL). These results indicate a consistent but limited antiviral effect of the Soxhlet extract against HSV-1.

*Table 2: Anti-HSV-1 Activity of Soxhlet Extract (S0144)*

| Concentration (µg/mL) | % HSV-1 Inhibition |
|---|---|
| 50.00 | 21.84 |
| 16.67 | 23.69 |
| 5.56 | 22.63 |
| 1.85 | **27.15** |
| 0.617 | 22.49 |
| 0.206 | 18.45 |

**Caption:**

*Antiviral activity of Soxhlet extract against HSV-1 in Vero cells.*

3.3 Antiviral Activity of Fermented Extract (S0145)

The fermented extract (S0145) exhibited consistently higher antiviral activity than the Soxhlet extract across most concentrations. The inhibition values ranged from 22.31% to 32.08%, with the maximum inhibition observed at 50 µg/mL (32.08%). Moderate inhibition was also maintained at lower concentrations, including 29.21% at 0.206 µg/mL, indicating sustained antiviral activity even under dilution conditions.

Similar to the Soxhlet extract, a strictly linear dose–response relationship was not observed; however, the fermented extract displayed a generally elevated inhibitory profile across the entire concentration range.

*Table 3: Anti-HSV-1 Activity of Fermented Extract (S0145)*

| Concentration (µg/mL) | % HSV-1 Inhibition |
|---|---|
| 50.00 | **32.08** |
| 16.67 | 24.64 |
| 5.56 | 25.82 |
| 1.85 | 25.57 |
| 0.617 | 22.31 |
| 0.206 | 29.21 |

**Caption:**
*Antiviral activity of fermented extract against HSV-1 in Vero cells.*

### 3.4 Comparative Analysis of Extraction Methods

A direct comparison between the two extraction methods revealed that the fermented extract consistently produced higher antiviral inhibition than the Soxhlet extract across most tested concentrations. While the Soxhlet extract demonstrated its highest inhibitory effect at lower concentration (1.85 µg/mL), the fermented extract achieved its maximum inhibition at the highest concentration (50 µg/mL). Overall, the fermented extract exhibited a broader and stronger antiviral inhibition profile, although neither extract reached the ≥50% inhibition threshold required to classify them as active antiviral agents.

*Table 4: Comparative Anti-HSV-1 Activity of Clinacanthus nutans Extracts*

| Concentration (µg/mL) | Soxhlet (% Inhibition) | Fermentation (% Inhibition) | Higher Activity |
|---|---|---|---|
| 50.00 | 21.84 | **32.08** | Fermentation |
| 16.67 | 23.69 | **24.64** | Fermentation |
| 5.56 | 22.63 | **25.82** | Fermentation |
| 1.85 | **27.15** | 25.57 | **Soxhlet** |
| 0.617 | 22.49 | 22.31 | Comparable |
| 0.206 | 18.45 | **29.21** | Fermentation |

**Caption:**
*Comparative antiviral performance of Soxhlet and fermented extracts against HSV-1 across multiple concentrations.*

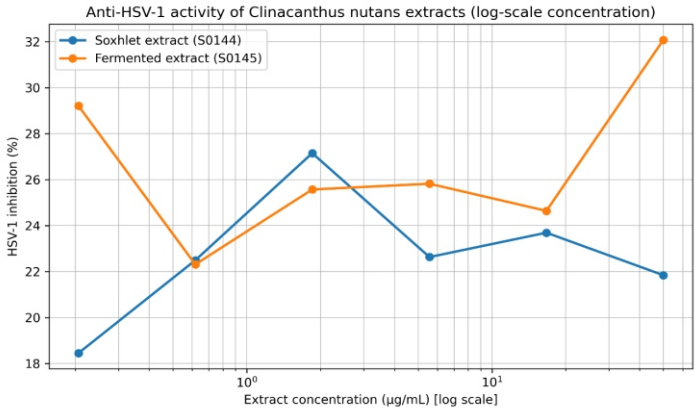


*Figure 2. Anti-HSV-1 activity of Clinacanthus nutans extracts*

**Caption** Anti-HSV-1 activity of *Clinacanthus nutans* leaf extracts obtained by Soxhlet extraction (S0144) and fermentation (S0145) across multiple concentrations (0.206–50 µg/mL) in Vero cells. Concentration is presented on a logarithmic scale ($\log_{10}$ µg/mL), and antiviral activity is expressed as percent viral inhibition.

### 3.5 Summary of Biological Activity

Taken together, the results demonstrate that both Soxhlet and fermentation extraction methods produce biologically active C. nutans extracts with measurable anti-HSV-1 effects and no detectable cytotoxicity in Vero cells. Fermentation enhanced antiviral activity across multiple concentrations, while Soxhlet extraction showed moderate inhibitory effects with notable activity at lower concentrations. These findings confirm that extraction strategy significantly influences the antiviral performance of C. nutans extracts.

## 4. Discussion

This study provides a comparative evaluation of the antiviral activity of *Clinacanthus nutans* leaf extracts obtained by Soxhlet extraction and fermentation against herpes simplex virus type-1 (HSV-1). Both extracts demonstrated measurable antiviral effects without detectable cytotoxicity in Vero cells, indicating that the observed viral inhibition was attributable to biological activity rather than extract-induced cellular damage. These findings support the biosafety profile of *C. nutans* extracts and validate their suitability for antiviral screening models.

Although neither extract reached the ≥50% inhibition threshold required for classification as a strongly active antiviral agent, both exhibited consistent moderate inhibitory effects across a wide concentration range. This pattern suggests the presence of bioactive constituents capable of interfering with HSV-1 replication, albeit at levels insufficient to produce high-potency antiviral effects in crude extract form. Such outcomes are consistent with many medicinal plant studies, where crude extracts often display moderate activity that can be substantially enhanced following fractionation and compound isolation.

A key finding of this study is the method-dependent difference in antiviral performance. The fermented extract consistently exhibited higher HSV-1 inhibition than the Soxhlet extract across most tested concentrations, indicating that fermentation enhanced antiviral potential. This enhancement may be explained by microbial biotransformation during fermentation, which can convert phytochemical precursors into more bioactive or more bioavailable forms. Previous studies have demonstrated that fermentation can increase the biological activity of plant extracts by modifying glycosylated compounds, releasing aglycones, or generating new metabolites with improved pharmacological properties. In contrast, Soxhlet extraction, while efficient and reproducible, primarily functions as a physicochemical extraction process that preserves the native phytochemical profile without biological modification.

Interestingly, the Soxhlet extract demonstrated its highest antiviral activity at a lower concentration (1.85 µg/mL), whereas the fermented extract achieved its maximal inhibition at the highest concentration (50 µg/mL). This non-linear concentration–response pattern suggests that multiple compounds may contribute to antiviral activity through different mechanisms and concentration-dependent interactions. Such behavior is typical of complex phytochemical mixtures, where synergistic and antagonistic interactions can obscure classical dose–response relationships observed in single-compound pharmacology.

The absence of significant cytotoxicity across all tested concentrations further strengthens the translational relevance of these findings. Safety is a critical factor in antiviral drug development, particularly for long-term or topical applications.

The demonstration that both extraction methods produce biologically active yet non-cytotoxic extracts supports the potential of *C. nutans* as a safe natural source for antiviral compound development.

From a broader perspective, this study highlights the importance of extraction strategy as a determinant of biological efficacy. Rather than viewing extraction as a purely technical process, the findings demonstrate that extraction methodology directly influences biological outcomes. Fermentation, in particular, emerges as a promising bioprocessing approach for enhancing antiviral potential in medicinal plants, offering a biologically driven method for activity optimization prior to chemical isolation.

Nevertheless, this study has limitations. The antiviral activity was evaluated using crude extracts, and no phytochemical profiling or compound identification was performed. As a result, the specific bioactive constituents responsible for HSV-1 inhibition remain unidentified. In addition, the absence of $IC_{50}$ determination limits quantitative pharmacological interpretation. Future studies should focus on phytochemical characterization using chromatographic and mass spectrometric techniques, bioactivity-guided fractionation, and mechanistic antiviral assays to identify active compounds and elucidate their modes of action.

In conclusion, this study demonstrates that both Soxhlet extraction and fermentation yield *C. nutans* extracts with measurable anti-HSV-1 activity and favorable safety profiles. Fermentation enhances antiviral performance relative to Soxhlet extraction, emphasizing the role of bioprocessing in optimizing biological activity. These findings provide a scientific foundation for future compound-level investigations and support the development of *C. nutans* as a potential natural source of antiviral agents.

## 5. Conclusion

This study demonstrates that *Clinacanthus nutans* leaf extracts obtained by both Soxhlet extraction and fermentation exhibit measurable anti-HSV-1 activity with favorable cellular safety profiles in Vero cells. Although the antiviral effects of both extracts remained within a moderate range and did not reach the threshold required for classification as highly active antiviral agents, clear method-dependent differences in biological performance were observed. Fermentation consistently enhanced antiviral inhibition compared with Soxhlet extraction across most tested concentrations, highlighting the importance of bioprocessing strategies in optimizing the biological potential of medicinal plant extracts. These findings establish a scientific foundation for extraction method–guided development of *C. nutans* as a source of antiviral bioactive compounds and support future investigations focused on phytochemical characterization, bioactivity-guided fractionation, and mechanistic antiviral studies to identify and validate active compounds for therapeutic development.

## 6. Declarations

### *6.1 Ethics Approval and Informed Consent*

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Institutional Review Board (IRB) of CADET-TUTOR SCHOOL (Approval No. 06003/2026).

### *6.2 Author Contributions*

The author developed the study concept and design, prepared the questionnaire, supervised data collection, analyzed and interpreted the data, and prepared the manuscript. The author reviewed and approved the final version of the manuscript.

### *6.3 Funding*

This research received no specific grant from any public, commercial, or non-profit funding agency.

### *6.4 Conflict of Interest*

The author declares no conflict of interest.

### *6.5 Data Availability Statement*

The data supporting the findings of this study are available from the corresponding author upon reasonable request. Data containing information that could affect participant confidentiality will not be publicly released.

### *6.6 Acknowledgments*

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