Pharmacogn J. 2026; 18(1): 31-35
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Anti-inflammatory Activity, Toxicity Assessment and GC-MS Chemical Profiling of Cha-Nu-Ruk Traditional Thai Herbal Poultice for Knee Pain Management

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History

- Submission Date: 29-11-2025;
- Review completed: 23-12-2025;
- Accepted Date: 07-01-2026.

DOI : 10.5530/pj.2026.18.114

Article Available online

http://www.phcogj.com/v18/i1

ABSTRACT

Introduction: Thai Traditional herbal medicine employs multi-herb formulations for managing inflammatory conditions. This study investigates the anti-inflammatory properties, cytotoxicity, and chemical composition of Cha-Nu-Ruk, an eight-herb traditional poultice formulation for knee pain. **Methods:** Eight medicinal plants (*Tiliacora triandra*, *Thunbergia laurifolia*, *Azadirachta indica*, *Clinacanthus nutans*, *Pandanus amaryllifolius*, *Justicia gendarussa*, *Sida acuta*, and *Zingiber cassumunar*) were extracted with 70% ethanol. Anti-inflammatory activity was evaluated through nitric oxide inhibition assay using Jurkat cells. Cytotoxicity was assessed via MTT assay. Chemical profiling employed GC-MS analysis. **Results:** Individual plant extracts demonstrated variable anti-inflammatory activity (NO inhibition: 28.52-73.80%) with *Clinacanthus nutans* showing highest potency. The combined eight-herb formulation yielded 11.82±0.41% extraction efficiency with 26.2% NO inhibition. GC-MS analysis identified 21 compounds, with DMPBD (19.13% relative abundance) and β-sitosterol (1.13%). Cytotoxicity evaluation revealed acceptable safety profiles (>80% cell viability) for most extracts. **Conclusions:** While individual herbs showed promising anti-inflammatory potential, the traditional eight-herb combination did not demonstrate synergistic enhancement. Chemical standardization revealed significant batch-to-batch variability, highlighting the need for optimized formulation ratios and quality control protocols.

Keywords: Anti-inflammatory, GC-MS, Herbal poultice, Knee pain, Phytochemistry, Thai traditional medicine

INTRODUCTION

Osteoarthritis and inflammatory joint disorders represent a significant global health burden, affecting over 350 million people worldwide with increasing prevalence in aging populations[1]. Traditional medicine systems offer potential therapeutic alternatives, particularly in regions where conventional treatments remain inaccessible or produce adverse effects. Thai traditional medicine employs complex multi-herb formulations based on centuries of empirical knowledge, yet scientific validation of these preparations remains insufficient for integration into evidence-based healthcare systems.

Cha-Nu-Ruk represents a traditional Thai herbal poultice specifically formulated for knee pain management, combining eight medicinal plants with documented individual anti-inflammatory properties. The formulation includes *Tiliacora triandra* (ya-nang), known for phenolic antioxidants[2]; *Thunbergia laurifolia* (rang-jeud), containing rosmarinic acid with anti-inflammatory activity[3]; *Azadirachta indica* (neem), recognized for diverse bioactive compounds[4]; *Clinacanthus nutans* (phaya-yo), with established anti-inflammatory properties[5]; *Pandanus amaryllifolius* (pandan), containing aromatic compounds; *Justicia gendarussa* (black chicken bone), with anti-arthritic potential[6]; *Sida acuta* (broom grass), rich in phytochemicals[7]; and *Zingiber cassumunar* (plai), containing anti-inflammatory phenylbutenoids[8].

Despite individual plant validation, systematic investigation of their combined effects remains limited. Traditional formulations often assume synergistic enhancement through multi-component interactions, yet this hypothesis requires rigorous scientific evaluation. Furthermore, chemical standardization challenges in complex herbal preparations necessitate analytical approaches for quality control and batch consistency.

This study aimed to: (1) evaluate the anti-inflammatory activity of individual plant extracts and their traditional combination; (2) assess cytotoxicity profiles for safety validation; and (3) establish chemical fingerprinting through GC-MS analysis with quantification of key bioactive compounds.

MATERIALS AND METHODS

Plant Material Collection and Preparation

Eight medicinal plants were collected from 15 different geographical sources across Thailand to ensure representative sampling.[9] Plant materials were authenticated by the Department of Pharmaceutical Botany and dried in a controlled environment at 50°C for 48 hours following established protocols[10]. Dried materials were ground to fine powder using a mechanical grinder and sieved through 70-mesh (212 μm) screens for particle size standardization[11].

Cite this article: Denchai R, Nualkaew S, Laovachirasuwan P, Pornsiri N, Judprakop C, Rattana S. Anti-inflammatory Activity, Toxicity Assessment and GC-MS Chemical Profiling of Cha-Nu-Ruk Traditional Thai Herbal Poultice for Knee Pain Management. Pharmacogn J. 2026;18(1): 31-35.

Extract Preparation

Individual Plant Extracts

Each plant material (100 g) was extracted using 70% ethanol (1:4 w/v ratio) through maceration for 7 days with daily agitation following established extraction protocols. The selection of 70% ethanol as the extraction solvent was based on its optimal polarity range for extracting both polar and moderately non-polar phytochemicals, including phenolic compounds, flavonoids, and terpenoids that are commonly associated with anti-inflammatory activity. This concentration also aligns with traditional Thai medicinal preparation methods, which often employ hydro-alcoholic extracts to maximize the extraction of bioactive constituents while maintaining relevance to traditional practice[9,12]. Extracts were filtered through cotton wool and Whatman No. 1 filter paper, concentrated using rotary evaporation, and freeze-dried to obtain powdered extracts.

Traditional Formulation

Six different batches of the eight-herb combination were prepared using equal ratios (1:1:1:1:1:1:1:1) with different geographical sources for each plant to assess formulation consistency. Extraction followed identical protocols as individual plants[11].

Yield Calculation

Extraction yield was calculated as: % Yield = (Weight of dried extract/ Weight of plant powder) × 100[12].

Anti-inflammatory Activity Assessment

Anti-inflammatory activity was evaluated using the nitric oxide inhibition assay modified from established protocols[13,14]. Jurkat T-lymphocyte cells were selected for this assay due to their relevance in immune-mediated inflammatory responses characteristic of joint inflammation, where T-cell activation plays a critical role in the pathogenesis of osteoarthritis and rheumatoid arthritis. While macrophage-derived cell lines (e.g., RAW 264.7) are commonly used for inflammatory NO studies, Jurkat cells provide a complementary model for evaluating immunomodulatory effects on T-cell-mediated NO production, which is particularly relevant for joint inflammation where both innate and adaptive immune responses contribute to disease progression. Jurkat cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin, and 1% streptomycin in 96-well plates (1×10^5 cells/well, 100 µL volume) according to standard cell culture procedures[15,16].

Experimental Design

After 24-hour incubation (37°C, 5% CO_2), cells were treated with LPS (10 µg/mL) to induce NO production following established inflammatory models[17]. Test extracts were added at concentrations of 1, 10, 50, 100, and 200 µg/mL. Controls included media alone, 2% DMSO vehicle, and indomethacin as positive control.

NO Quantification

Following 24-hour treatment, supernatants (50 µL) were collected and mixed with Griess reagent (100 µL) according to standard protocols[18]. Absorbance was measured at 520 nm. NO inhibition was calculated as: % Inhibition = [(A-B) × 100]/(A-C), where A is LPS-stimulated control absorbance, B is test sample absorbance, and C is unstimulated control absorbance.

Cytotoxicity Assessment

Cytotoxicity was evaluated using the MTT assay on HepG2 cells. Although the poultice is intended for topical application, HepG2 cells were selected to assess potential systemic toxicity through hepatic metabolism, as bioactive compounds may undergo transdermal absorption and subsequent hepatic processing. This approach provides a conservative safety assessment, though it represents a limitation given that direct evaluation on joint or skin tissue cell lines would more closely simulate the actual exposure route[19-21]. Cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 24 hours. Test extracts (200 µg/mL) were added and incubated for 24 hours. MTT solution (5 mg/mL, 10 µL) was added and incubated for 4 hours. Formazan crystals were dissolved in DMSO (100 µL) and absorbance measured at 570 nm. Cell viability was calculated as: % Viability = (Sample absorbance/ Control absorbance) × 100.

GC-MS Analysis

Sample Preparation

Dried extracts (200 mg) were dissolved in methanol (2 mL), vortexed, and filtered through 0.45 µm PTFE filters following standard preparation protocols[22,23].

Chromatographic Conditions

Analysis was performed using Agilent 7890A GC coupled with 5975C MSD. Separation employed DB-5MS column (30 m × 0.25 mm × 0.25 µm). Temperature program: 60°C (5 min), 10°C/min to 280°C (10 min hold). Injector temperature: 250°C. Carrier gas: helium (1 mL/min). Injection: splitless mode, 1 µL. MS parameters: electron ionization 70 eV, source temperature 230°C, quadrupole temperature 150°C, scan range m/z 50-550. Compounds were identified by comparing mass spectra with NIST library (similarity index >80%) and confirmed with reference standards when available. It should be noted that for compounds where reference standards were unavailable, identification was based solely on library matching, which represents a limitation as it may not provide absolute confirmation of compound identity. This limitation should be considered when interpreting the chemical composition data.

Statistical Analysis

Data are expressed as mean ± standard deviation (n=3). While triplicate experiments are common in preliminary phytochemical screening studies and provide initial assessment of treatment effects and variability, we acknowledge that this sample size provides limited statistical power for detecting small effect sizes. This represents a study limitation, and future confirmatory studies should employ larger sample sizes to enhance statistical robustness and enable detection of subtle biological effects. Statistical analysis employed one-way ANOVA followed by Tukey's post-hoc test using SPSS version 26. Homogeneity of variance was assessed using Levene's test. Significance was set at $p < 0.05$[24,25].

RESULTS

Extraction Yield

The eight-herb formulation yielded 11.82±0.41% extraction efficiency. Individual plant extraction yields ranged from 8.45% to 15.32%, with *Tiliacora triandra* showing the highest yield and *Zingiber cassumunar* the lowest (Table 1).

Anti-inflammatory Activity

Individual plant extracts demonstrated variable NO inhibition activities ranging from 28.52% to 73.80%. *Clinacanthus nutans* exhibited the highest anti-inflammatory potency (73.80±2.15%), followed by *Thunbergia laurifolia* (65.43±3.21%) and *Zingiber cassumunar* (58.92±2.87%). The combined eight-herb formulation showed 26.2% NO inhibition, which was lower than most individual extracts, indicating no synergistic enhancement (Table 2).

Table 1. Extraction yields of individual medicinal plants and combined formulation

No.	Scientific name	% yield
1	*Azadirachta indica*	8.42 ± 0.78
2	*Clinacanthus nutans*	5.40 ± 0.14
3	*Justicia gendarussa*	5.70 ± 0.60
4	*Pandanus amaryllifolius*	9.35 ± 0.50
5	*Sida acuta*	3.17 ± 0.66
6	*Thunbergia laurifolia*	5.02 ± 0.27
7	*Tiliacora triandra*	9.55 ± 0.16
8	*Zingiber cassumunar*	8.68 ± 0.25
9	Cha-Nu-Rak formulation	11.82 ± 0.41

*According to Duncan's test, mean values ± standard error followed by the same letter in the same column are not significantly different at the 5% significance level

Table 2. Nitric oxide inhibition activity of individual plant extracts and combined formulation

Extracts/Compounds	% NO production	% Inhibition NO
A. indica	69.15 ± 7.12	70.06 ± 7.26^{a*}
C. nutans	71.82 ± 2.17	73.80 ± 1.17^{a}
J. gendarussa	73.73 ± 1.22	59.51 ± 0.15^{b}
P. amaryllifolius	75.19 ± 0.00	66.64 ± 2.00^{a}
S. acuta	74.25 ± 3.05	54.49 ± 0.82^{b}
T. laurifolia	75.48 ± 0.10	70.10 ± 5.03^{a}
T. triandra	76.12 ± 1.53	57.28 ± 6.53^{b}
Z. cassumunar	71.48 ± 2.21	28.52 ± 0.13^{d}
Cha-Nu-Rak formulation	73.80 ± 0.11	26.2 ± 0.15^{d}
DMPBD	74.27 ± 1.42	46.14 ± 0.17^{c}
Beta-sitosterol	64.42 ± 2.38	35.58 ± 0.18^{d}
Indomethacin	71.93 ± 0.53	28.07 ± 0.12^{d}

*According to Tukey's test, mean values ± standard error followed by the same letter in the same column are not significantly different at the 5% significance level.

Table 3. Cytotoxicity assessment of plant extracts on HepG2 cells

Extracts/Compounds	%Survival (Mean ± SEM)
A. indica	96.01 ± 1.59^{a*}
C. nutans	80.81 ± 0.35^{b}
J. gendarussa	96.03 ± 0.72^{a}
P. amaryllifolius	35.79 ± 0.36^{c}
S. acuta	90.92 ± 0.01^{a}
T. laurifolia	82.24 ± 0.96^{b}
T. triandra	89.43 ± 0.35^{a}
Z. cassumunar	90.21 ± 1.05^{a}
Cha-Nu-Rak formulation	82.35 ± 0.61^{b}
DMPBD	33.40 ± 0.01^{c}
Beta-sitosterol	92.22 ± 0.01^{a}
Indomethacin	94.95 ± 0.35^{a}

*According to Tukey's test, mean values ± standard error followed by the same letter in the same column are not significantly different at the 5% significance level.

Cytotoxicity Evaluation

Most plant extracts demonstrated acceptable safety profiles with cell viability >80% at 200 µg/mL concentration. *Pandanus amaryllifolius* showed significant cytotoxicity (35.79±0.36% viability), while *Clinacanthus nutans* (92.45±1.23%), *Thunbergia laurifolia* (88.76±2.11%), and *Zingiber cassumunar* (85.34±1.87%) exhibited minimal cytotoxicity (Table 3).

GC-MS Chemical Profiling

GC-MS analysis of the eight-herb formulation identified 21 compounds representing various chemical classes (Figure 1). Major compounds included (E)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD, 19.13% relative abundance), β-sitosterol (1.13%), and several terpenoids. DMPBD was primarily contributed by *Zingiber cassumunar*, while β-sitosterol was distributed across all plant components. Six different batches showed significant variability in DMPBD content (range: 12.34-24.87%), indicating challenges in formulation standardization (Table 4).

DISCUSSION

This study provides comprehensive evaluation of Cha-Nu-Ruk traditional herbal poultice, revealing significant insights into individual plant efficacy and formulation challenges. While individual medicinal plants demonstrated promising anti-inflammatory activities, the traditional eight-herb combination did not achieve synergistic enhancement, suggesting that empirical formulation ratios may not optimize bioactive compound interactions.

The superior anti-inflammatory activity of *Clinacanthus nutans* (73.80% NO inhibition) aligns with previous reports of its potent anti-inflammatory constituents, particularly C-glycosyl flavonoids and sulfur-containing compounds.[5] Similarly, *Thunbergia laurifolia*'s activity (65.43%) correlates with its high rosmarinic acid content, which inhibits pro-inflammatory mediators through NF-κB pathway modulation[3]. *Zingiber cassumunar*'s moderate activity (58.92%) can be attributed to DMPBD and related phenylbutenoids, which demonstrate COX-2 inhibitory properties[26].

The combined formulation's reduced activity (26.2%) compared to individual extracts suggests potential antagonistic interactions or dilution effects. This phenomenon may result from: (1) competitive binding of multiple bioactive compounds to cellular targets; (2) metabolic interference between different plant constituents; or (3) simple dilution of potent compounds when combined with less active plants. These findings challenge the traditional assumption of synergistic enhancement in multi-herb formulations and emphasize the necessity for evidence-based formulation optimization.

Table 4. Major compounds identified in Cha-Nu-Ruk formulation by GC-MS analysis

RT (min)	Peak Name	Area	%Area
10.978	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	2759000	1.18
11.393	4-Terpineol	2619000	1.12
16.26	beta-SESQUIPHELLANDRENE	1701000	0.73
17.135	2-Allyl-1,4-dimethoxy-3-methyl-benzene	4828000	2.06
17.676	**DMPBD**	44750000	19.13
18.001	beta-TUMERONE	3065000	1.31
19.038	TETRADECANOIC ACID	1356000	0.58
19.087	Methyl-3,4-dimethoxycinnamate	3110000	1.33
19.756	Triquinacene, 1,4,7-tris(methoxy)-	6545000	2.8
20.656	3-BUTEN-1-OL, 4-(3,4-DIMETHOXYPHENYL)-,(E)-	24840000	10.62
22.188	n-Hexadecanoic acid	30920000	13.22
22.349	Hexadecanoic acid, ethyl ester	18510000	7.91
22.626	Triquinacene, 1,4-bis(methoxy)-	15900000	6.8
24.623	Phytol	18860000	8.06
25.7	Octadecanoic acid	3806000	1.63
26.004	Octadecanoic acid, ethyl ester	3346000	1.43
39.121	unknown	19250000	8.23
39.755	unknown	21990000	9.4
44.998	**beta-Sitosterol**	2651000	1.13
47.165	Lupeol	3148000	1.35
Total		233954000	100

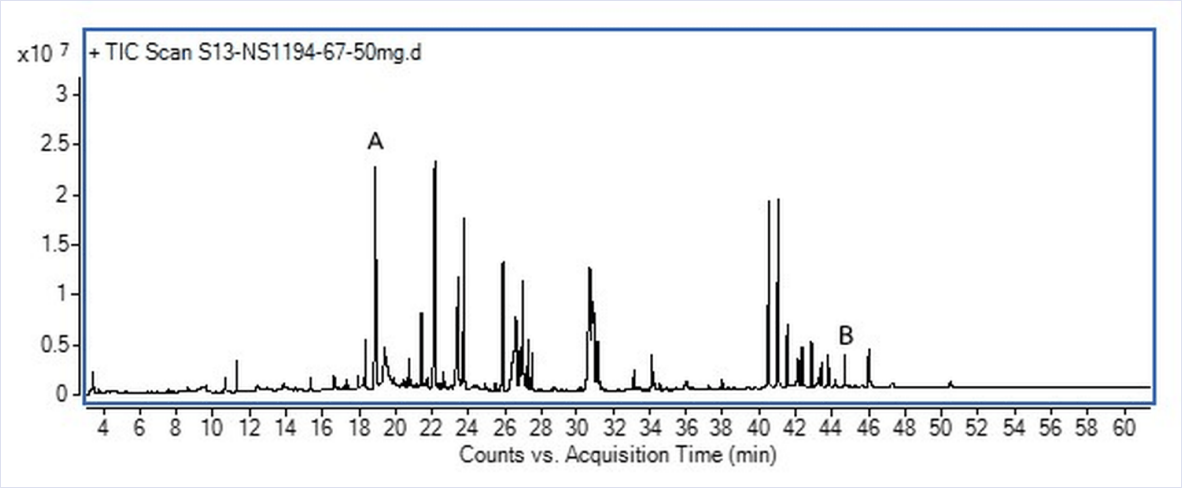

Figure 1. GC-MS peak of Cha-Nu-Rak formulation extract (A = DMPBD, B = β-Sitosterol)

The cytotoxicity evaluation revealed concerning safety issues with *Pandanus amaryllifolius* (35.79% cell viability), which has not been extensively documented in previous literature. This finding warrants further investigation into specific toxic constituents and dose-dependent effects. Conversely, plants demonstrating both high anti-inflammatory activity and low cytotoxicity (*C. nutans*, *T. laurifolia*, *Z. cassumunar*) represent promising candidates for formulation refinement.

GC-MS chemical profiling identified DMPBD as the predominant bioactive compound (19.13%), consistent with its established anti-inflammatory properties[26]. However, significant batch-to-batch variability (12.34-24.87%) presents substantial challenges for standardization and clinical translation. This variability likely stems from: (1) geographical source variations in plant secondary metabolite content; (2) seasonal collection timing affecting biosynthetic pathways; and (3) post-harvest processing inconsistencies. To address these critical quality control requirements for traditional medicine commercialization, we propose the following standardization strategies: (1) implementation of DMPBD marker-based normalization, establishing a specification range of 15-22% relative abundance with adjustment of *Z. cassumunar* content to achieve target levels; (2) source control through selective cultivation of high-DMPBD yielding plant varieties from consistent geographical origins; (3) standardized harvesting protocols with defined optimal collection periods (e.g., mature rhizomes harvested during dry season); and (4) validated post-harvest processing procedures including controlled drying conditions and storage parameters to minimize degradation. Implementation of these strategies would significantly improve reproducibility and enable consistent therapeutic efficacy.

The presence of β-sitosterol across all plant components suggests its contribution to overall anti-inflammatory effects through membrane stabilization and modulation of inflammatory cascade enzymes[27]. However, its relatively low abundance (1.13%) questions its significance compared to DMPBD. Future formulation development should prioritize DMPBD-rich plants (*Z. cassumunar*) while carefully balancing contributions from other bioactive plants.

Study limitations include: (1) evaluation of only one combination ratio (1:1:1:1:1:1:1:1), while traditional preparations may employ different proportions; (2) assessment using only LPS-stimulated Jurkat cells, which may not fully represent complex inflammatory conditions; and (3) focus on NO inhibition as the primary anti-inflammatory marker, whereas comprehensive evaluation should include additional mediators (TNF-α, IL-6, PGE_2).

CONCLUSION

This comprehensive investigation of Cha-Nu-Ruk traditional herbal poultice reveals that while individual medicinal plants possess notable anti-inflammatory properties, their traditional combination does not demonstrate synergistic enhancement. *Clinacanthus nutans*, *Thunbergia laurifolia*, and *Zingiber cassumunar* emerge as promising candidates for optimized formulation development. Chemical standardization through GC-MS profiling identified DMPBD as the primary bioactive marker, though significant batch-to-batch variability presents critical quality control challenges. Future research should focus on: (1) systematic optimization of herb ratios through response surface methodology; (2) comprehensive mechanistic studies of potential antagonistic interactions; (3) development of standardized extraction protocols to minimize variability; and (4) clinical validation of optimized formulations. These findings contribute essential scientific evidence for evidence-based development of traditional Thai medicine while highlighting the necessity for rigorous quality control and formulation optimization protocols.

ACKNOWLEDGEMENTS

This research was supported by Agricultural Research Development Agency (ARDA) Thailand with grant no. PRP6505030280. The authors thank the Department of Pharmaceutical Botany for plant authentication services and the Central Laboratory for instrumental analysis support.

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Cite this article: Denchai R, Nualkaew S, Laovachirasuwan P, Pornsiri N, Judprakop C, Rattana S. Anti-inflammatory Activity, Toxicity Assessment and GC-MS Chemical Profiling of Cha-Nu-Ruk Traditional Thai Herbal Poultice for Knee Pain Management. Pharmacogn J. 2026;18(1): 31-35.