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Comparative phytochemicals and biological activities of ethanolic extracts of *Clinacanthus nutans* leaves and validation of HPLC method for vitexin

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ABSTRACT: Phaya Yo (*Clinacanthus nutans*) leaves have been traditionally used in Thailand to treat insect stings and herpes virus infections. Their efficacy is recognized, leading to the inclusion of a 95% ethanolic extract in the Thai National Essential Medicine List. The plant's key active compounds are apigenin-C-glycoside flavones, including vitexin, iso-vitexin, orientin, and schaftoside, though their levels vary due to environmental factors and extraction methods. This study examined different drying methods—oven drying, parabolic dome drying, and sun drying—followed by maceration using 95%, 70%, and 50% hydro-alcoholic solvents. Results showed that oven drying yielded the highest antioxidant activity, total phenolic content (TPC), and total flavonoid content (TFC). The 95% ethanolic extract outperformed the others in antioxidant activity, TPC, TFC, and inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells. HPLC analysis confirmed the presence of vitexin but not schaftoside, with the highest vitexin content (308.67 ± 1.36 µg/g dry weight) found in the 95% ethanolic extract. These findings support the use of oven-dried *C. nutans* leaves extracted with 95% ethanol for optimal bioactivity. This study also provides an improved HPLC system for analyzing apigenin-C-glycoside flavones in *C. nutans* leaf extract.

KEYWORDS: antioxidant, total flavonoid content, total phenolic content, anti-inflammatory, RAW 264.7 cells

INTRODUCTION

Clinacanthus nutans (Burm.f.) Lindau, a small shrub from the Acanthaceae family, is a well-known traditional herb in Southeast Asia. In Thailand, it is called Phaya Yo or Snake Grass in English and grows in mixed deciduous forests or home gardens. Thai folk medicine uses fresh *C. nutans* leaves blended with rice whisky (40% ethanol) to treat insect bites, stings, and herpes lesions [1]. Recognizing its efficacy, Phaya Yo ointment, containing 4–5% w/w of a 95% ethanolic extract, has been included in the Thai National Essential Medicine List for relieving inflammation, pain, and swelling from insect bites and stings. Later, the extract from *C. nutans* leaves has been shown to possess analgesic, anti-inflammatory, and antiviral activities against Varicella zoster virus (VZV) and Herpes simplex virus (HSV) type-2 [2, 3], antioxidant activity, and protective effect against free radical-induced hemolysis [4].

The chemical composition of *C. nutans* leaf ethanol extract as analyzed by LC-MS and MS/MS led to identification of several compounds, including myricetin, orientin, iso-orientin, vitexin, iso-vitexin, iso-okanin, apigenin, and ferulic acid [5]. Studies have shown that vitexin exhibits analgesic effects in inflammatory pain models [6] and improves brain cell ultrastructure in D-galactose-aged mice [7]. It also demonstrates antinociceptive properties via opioid and GABA receptors [8] and significantly reduces the production of NO and TNF-α in human neutrophils by 86.74% and 80.94%, respectively, at 25 µM. Additionally, vitexin has been reported to have antioxidant [9], antibacterial [10], antiviral [11], and antinociceptive [12] activities. A previous study on a *C. nutans* leaf extract containing schaftoside, vitexin, and isovitexin as major constituents was evaluated in ovariectomized rats and compared with diclofenac. Assessment of serum pro-inflammatory markers (NF-κB, COX-2, and IL-1β) showed that a dose of 400 mg/kg *C. nutans* extract produced anti-inflammatory effects comparable to diclofenac for preventing osteoporotic osteoarthritis [13].

Vitexin, a flavonoid glycoside (Fig. 1), was chosen as the chemical marker for *C. nutans* due to its high stability and pharmacological relevance [1]. Because phytochemical content and biological activity vary with environmental factors [14], extraction methods, and solvent choice, this study employed simple maceration, which has been shown to yield the highest antioxidant activity in the DPPH assay [15]. Extraction is a mass transfer process in which molecules move

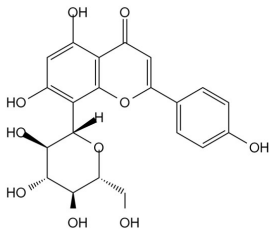

Fig. 1 Structure of vitexin.

from a solid matrix into a liquid solvent. It involves two steps: rapid solvent penetration into the solid, influenced by stirring, temperature, and solvent-to-solid ratio, followed by diffusion of solutes into the solvent according to Fick's law, governed by solvent properties, temperature, and extraction time. These principles justify the use of different hydroalcoholic solvents in this study [16].

This study aims to evaluate the total phenolic and flavonoid content, antioxidant activity, *in vitro* anti-inflammatory effects, and vitexin levels in *C. nutans* extracts prepared using different drying methods and ethanol concentrations (95%, 70%, and 50%). A validated HPLC method was developed for vitexin quantification. The findings will help identify optimal drying and extraction conditions for *C. nutans* as an antioxidant and anti-inflammatory agent and support its future applications.

MATERIALS AND METHODS

Plant materials

The Phaya Yo leaves were collected in Suphan Buri Province, Thailand, on January 13, 2024. The specimen was kindly identified as *Clinacanthus nutans* (Burm. f.) Lindau by Dr. Sunisa Sangvirojanapat (the Botanist at the SireeruckhachatiNatureLearning 74 Park, Mahidol University (MU)) and deposited in the MU Herbarium under voucher PBM No. 006310.

Chemicals and reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), ascorbic acid, and gallic acid were purchased from Merck (Darmstadt, Germany). Quercetin hydrate (⩾95%), Folin–Ciocalteu reagent, aluminum chloride (reagent grade, 98%), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), dexamethasone (Dex), thiazolyl blue tetrazolium bromide (MTT; ⩾98%), potassium persulfate ($K_2S_2O_8$; ACS reagent, ⩾99.0%) and lipopolysaccharide (LPS; *Escherichia coli* O111:B4) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ, ⩾98%) and vitexin (analytical standard) were purchased from Supelco (Bellefonte, PA, USA). The murine macrophage cell line RAW 264.7 (TIB-71) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Instruments

RP-HPLC system (Thermo Scientific UltiMate™ 3000, Thermo Fisher Scientific, Waltham, MA, USA); microplate reader (EnSight™Multimode Plate Reader, PerkinElmer, Inc., Waltham, MA, USA); hot air oven (Memmert UN 260, Memmert GmbH + Co. KG, Schwabach, Germany).

Drying methods

C. nutans leaves were dried using three methods: oven drying, sun drying under a net, and greenhouse solar drying in a polycarbonate parabolic dome without temperature control. Drying continued until constant weight was reached. Each method began at 8:30 AM and used three 100-g fresh leaf replicates, with temperature and relative humidity recorded throughout.

Preparation of the extracts

A comparison study of Soxhlet extraction, simple maceration, and kinetic maceration using ethanol showed that simple maceration produced the strongest DPPH antioxidant activity ($IC_{50} = 14.20 \pm 0.49$ mg/ml) [15]; therefore, this method was selected for the study. A 10:1 liquid-to-solid (L/S) ratio enhances extraction efficiency compared with 5:1 by maintaining a stronger concentration gradient, improving solute diffusion, and preventing solvent saturation. The larger solvent volume also promotes better wetting and interaction with the solid matrix, increasing mass transfer, and extraction yield [17]. Therefore, the powdered 100 g samples *C. nutans* leaves from each drying method were macerated with 95%, 70%, or 50% ethanol ($n = 3$) at a 10:1 L/S ratio for three days [18]. Extracts were then filtered, vacuum-evaporated, and freeze-dried.

Evaluation of antioxidant activities

Determination of antioxidant activity of *C. nutans* leaves by DPPH assay

DPPH radical scavenging activity was assessed following a standard method [19]. A 152 µM DPPH methanolic solution was prepared, and extracts were diluted to 156.25–2,500 µg/ml. In a 96-well plate, 100 µl of extract was mixed with 100 µl of DPPH solution. The control contained DPPH with methanol, and the blank contained methanol with sample. After 30 min of dark incubation at room temperature, absorbance (A) at 517 nm was measured in triplicate, and inhibition (%) was calculated using Eq. (1).

$$\%\,\text{Radical scavenging} = \frac{A_{\text{control DPPH}} - A_{\text{sample DPPH}}}{A_{\text{control DPPH}}} \times 100 \quad (1)$$

Ascorbic acid served as the standard, and antioxidant activity was expressed as the IC_{50} value derived from the linear calibration curve of extract concentration versus % radical scavenging.

Determination of antioxidant activity of *C. nutans* leaves by ABTS assay

The ABTS cation radical decolorization assay was performed following a published method [20]. $ABTS^{\bullet+}$ was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate and incubating the mixture in the dark for 12–16 h. Before use, the solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Extracts were prepared at 156.25–2,500 µg/ml in methanol. In a 96-well plate, 20 µl of sample was mixed with 180 µl of $ABTS^{\bullet+}$ solution; blanks contained ethanol instead of $ABTS^{\bullet+}$. After 10 min of dark incubation at room temperature, absorbance (A) at 734 nm was measured in triplicate, and inhibition (%) was calculated using Eq. (2).

$$\%\ \text{ABTS radical scavenging} = \frac{A_{\text{control ABTS}} - A_{\text{sample ABTS}}}{A_{\text{control ABTS}}} \times 100 \quad (2)$$

Ascorbic acid was used as the standard, and antioxidant activity expressed as the IC_{50} value was obtained as described in DPPH assay.

Determination of TPC

TPC was measured using the Folin–Ciocalteu method [19] with gallic acid as the standard. A 5 mg/ml extract solution was prepared, and 20 µl was mixed with 100 µl of Folin–Ciocalteu reagent diluted 1:10 with deionized water. After 3 min, 80 µl of 7.5% Na_2CO_3 was added and incubated for 30 min in the dark. Absorbance at 765 nm was measured in triplicate, and TPC was calculated from a gallic acid calibration curve (25–400 µg/ml) and expressed as µg GAE/mg extract.

Determination of TFC

TFC was measured using a published method [21] with quercetin as the standard. A 5 mg/ml extract solution was prepared, and 100 µl was mixed with 100 µl of 2% $AlCl_3$. After 10 min of dark incubation at room temperature, absorbance at 415 nm was measured in triplicate. TFC was calculated from the quercetin calibration curve (10–45 µg) and expressed as µg QE/mg extract.

Determination of cell viability

The MTT assay was performed following published methods [22] with minor modifications. RAW 264.7 cells were treated with *C. nutans* leaf extracts prepared in 95%, 70%, or 50% ethanol. Extracts were diluted in DMSO, keeping the final DMSO concentration at 0.1% in all wells. Cells (5×10^4 cells/well) were seeded in 96-well plates and incubated at 37 °C with 5% CO_2 for 24 h before treatment with extracts at 25, 50, 100, and 200 µg/ml for an additional 24 h. After incubation, MTT was added and cells were incubated for 3 h, followed by dissolution of formazan with 100 µl DMSO. Absorbance was measured at 550 nm. Cells treated with 0.1% DMSO served as the 100% viability control, and cell viability was calculated using Eq. (3).

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (3)$$

Measurement of NO production inhibition

Nitrate and nitrite levels were measured using the Griess reagent with a NO assay kit following the described method [23]. RAW 264.7 cells (5×10^4 cells/well) were seeded in 96-well plates overnight, pretreated with 95%, 70%, or 50% ethanol extracts for 1 h, and then stimulated with LPS (100 ng/ml) for 24 h. Dexamethasone (40 µM) served as the positive control. After incubation, 100 µl of supernatant was collected, and nitrite levels were quantified using the Griess reaction, based on a sodium nitrite calibration curve. NO production inhibition was expressed as a percentage using Eq. (4).

$$\text{Percentage of NO inhibition (\%)} = \frac{A_{\text{LPS}} - A_{\text{Sample}}}{A_{\text{LPS}}} \times 100 \quad (4)$$

where A_{Sample} = absorbance of sample-treated cell and A_{LPS} = absorbance of only LPS-treated cell.

Method validation and determination of vitexin in *C. nutans* leaf extracts by high performance liquid chromatography (HPLC)

Instruments and chromatographic conditions

HPLC analysis was conducted using a Thermo Scientific UltiMate™3000 with a PDA detector and automatic injector, controlled by Chromeleon™software. Separation was performed on a Thermo Acclaim™ 120 C18 column (250 mm×4.6 mm, 5 µm). The mobile phase consisted of 1.0% acetic acid in water (solvent A) and acetonitrile (solvent B), using a gradient elution program as follows: 0 min, 95:5; 5 min, 90:10; 10 min, 85:15; 25 min, 70:30; 30 min, 0:100; and 35 min, 95:5 (A:B). The flow rate was 0.7 ml/min, run time 35 min, detection at 330 nm, and injection volume of 10 µl. Solvents were filtered (0.45 µm) and degassed before use.

Preparation of vitexin standard and sample solution

A 600 µg/ml vitexin stock solution was prepared by dissolving 3 mg of vitexin in 5 ml methanol and diluted to 1.875–60 µg/ml. All solutions were filtered through a 0.45 µm nylon filter before injection. For sample preparation, 100 mg of each extract (95%, 70%, and 50% freeze-dried ethanolic extracts) was dissolved in 5 ml methanol (20 mg/ml). A 2.5 ml aliquot was then diluted to 5 ml to obtain a 10 mg/ml analytical solution, which was filtered prior to HPLC analysis ($n = 3$).

Method validation

Method validation followed ICH guidelines, assessing system suitability, accuracy, precision, LOD, LOQ,

and linearity. Linearity was evaluated at six vitexin concentrations (1.875–60 µg/ml), each injected five times. Calibration curves were constructed by plotting concentration versus peak area, and linearity was determined using least-squares regression, with an acceptable $R^2 \geqslant 0.9950$ [24]. LOD and LOQ were calculated from the calibration curve using Eq. (5), where σ is the standard deviation of the y-intercepts and s is the slope.

$$\text{LOD} = (3.3x\sigma)/s; \qquad \text{LOQ} = (10x\sigma)/s \qquad (5)$$

Precision was evaluated at 7.5, 15, and 30 µg/ml by repeated injections. Intra-day precision was assessed using five replicates in one day, and inter-day precision using five replicates over three days. Precision was expressed as %RSD, which was required to be ⩽2%. Accuracy was determined through recovery tests by spiking samples with vitexin at 7.5, 15, and 30 µg/ml and analyzing the recovered amounts.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 23, and differences were evaluated by one-way ANOVA with significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Effects of drying methods

The Parabolic dome generated the highest drying temperature (50–60 °C) due to the greenhouse effect, followed by the hot-air oven at 50 °C, while sun drying produced the lowest temperature (30–45 °C). The hot-air oven showed the lowest relative humidity (15–22%), followed by the Parabolic dome (25–40%), whereas sun drying had the highest (35–50%). Both the Parabolic dome and the hot-air oven required approximately 4 h for drying, whereas sun drying took the longest (about 7.5 h) due to its lower temperature and higher humidity (Table 1).

Effects of different extraction solvents

Extraction with 50% ethanol produced the highest yield (11.84%), followed by 70% ethanol (11.23%), while 95% ethanol gave the lowest yield (10.65%) (Table 1). This likely reflects the ability of the aqueous component to extract more polar, non-flavonoid constituents such as sugars.

Antioxidant activity

The antioxidant activity of *C. nutans* extracts was evaluated using DPPH and ABTS assays. In the DPPH assay, antioxidants reduce the DPPH radical to its non-radical form, causing a color change from purple to yellow [25, 26]. The strongest DPPH scavenging activity was observed in the 95% ethanolic extract from oven-dried leaves (IC_{50} = 316.01 ± 5.29 µg/ml), followed by the 70% and 50% ethanolic extracts (452.69 ± 13.57 and 1,066.26 ± 39.29 µg/ml, respectively). Ascorbic acid showed an IC_{50} of 2.41 ± 0.11 µg/ml (Table 2).

ABTS oxidation with potassium persulfate forms the $ABTS^{\bullet+}$ radical, which is reduced by hydrogen-donating antioxidants, leading to decolorization of the blue radical cation [27, 28]. Ascorbic acid, the positive control, showed strong activity (IC_{50} = 2.78 ± 0.03 µg/ml). The 95% ethanolic extract exhibited the highest $ABTS^{\bullet+}$ scavenging activity (IC_{50} = 100.80 ± 5.88 µg/ml), followed by the 50% and 70% ethanolic extracts (102.22 ± 8.07 and 181.37 ± 11.44 µg/ml, respectively) (Table 2).

The ABTS assay produced lower IC_{50} values than the DPPH assay, consistent with previous reports showing IC_{50} ranges of 476.30 ± 0.74 to 1,024.00 ± 4.18 µg/ml for ABTS and 560.50 ± 2.45 to 1,530.00 ± 3.74 µg/ml for DPPH [29]. Earlier work also found stronger DPPH scavenging activity in 100% ethanolic extracts than in 70% ethanolic extracts [30]. Another study using crude methanolic extract and its dichloromethane and hexane fractions reported IC_{50} values of 560.50 ± 2.45, 1,039.00 ± 0.87, and 1,530.00 ± 3.74 µg/ml, respectively [29].

TPC and TFC

The 95% ethanolic extract of *C. nutans* leaves showed the highest TPC (66.04 ± 5.12 µg GAE/mg), significantly greater than the 70% and 50% ethanolic extracts (49.66 ± 2.85 and 42.35 ± 5.48 µg GAE/mg, respectively) (Table 2). Similarly, TFC was highest in the 95% ethanolic extract from oven-dried leaves (47.41 ± 1.93 µg QE/mg), followed by extracts from parabolic-dome drying (40.53 ± 8.77 µg QE/mg) and sun drying (33.19 ± 7.69 µg QE/mg). These values are much higher than those reported for *C. nutans* extracts from Malaysia and Indonesia, which showed markedly lower TPC and TFC [5, 18]. A similar study also found that oven drying at 50 °C produced the highest phenolic and flavonoid levels [31]. In general, higher TPC and TFC correlate with stronger antioxidant activity, consistent with the superior activity of the 95% ethanolic extract in this study (Table 2). Previous work has identified C-glycosyl flavones such as vitexin, isovitexin, and schaftoside in *C. nutans* [32, 33], which contribute significantly to its antioxidant properties.

Effect of ethanolic extracts of *C. nutans* on RAW 264.7 cell viability

MTT assays were used to identify non-cytotoxic concentrations of the extracts prior to anti-inflammatory testing. Extract concentrations of 31.25–1,000 µg/ml maintained RAW 264.7 cell viability above 80%. At 1,000 µg/ml, viability was 86.88 ± 2.62% for the 95% extract, 103.09 ± 6.22% for the 70% extract, and 92.39 ± 4.89% for the 50% extract. Based on previous criteria, cell viability above 80% indicates non-toxicity to RAW 264.7 cells [34].

Table 1 Percentage yield of *C. nutans* extracts in different hydroalcoholic solvents under different drying methods, temperature, relative humidity, duration and loss on drying.

Drying method	Percentage of extract (% w/w)			Temperature	% Relative	Time (h)/% loss
	95% Ethanol	70% Ethanol	50% Ethanol	(°C)	humidity	on drying
Oven	10.65 ± 0.22^{aA}	11.23 ± 0.52^{abA}	11.84 ± 0.21^{bA}	50	15–22	$4.0/6.96 \pm 0.14$
Sun	12.33 ± 0.29^{aB}	12.23 ± 0.34^{aB}	14.41 ± 0.40^{bB}	30–45	35–50	$7.5/9.00 \pm 0.04$
Parabolic dome	14.93 ± 0.25^{aC}	13.09 ± 0.39^{bC}	13.79 ± 0.24^{cC}	50–60	25–40	$4.0/8.22 \pm 0.15$

Different superscripts, $^{a–c}$within a row, $^{A–C}$within a column, are significantly different ($p < 0.05$) using Duncan's multiple range test.

Table 2 Effect of drying methods and different hydroalcoholic solvents on antioxidant activities by DPPH, and ABTS assays, TPC and TFC in *C. nutans* leaves.

Analysis	Drying method	Extract		
		95% Ethanol	70% Ethanol	50% Ethanol
DPPH scavenging activity	Oven	316.01 ± 5.29^{aA}	452.69 ± 13.57^{bA}	$1{,}066.26 \pm 39.29^{cA}$
(IC_{50} µg/ml)	Sun	579.46 ± 6.91^{aB}	846.69 ± 16.97^{bB}	$1{,}436.62 \pm 10.65^{cB}$
	Parabolic dome	440.91 ± 4.67^{aC}	869.13 ± 21.12^{bB}	$1{,}419.47 \pm 41.52^{cB}$
ABTS radical scavenging activity	Oven	100.80 ± 5.88^{aA}	181.37 ± 11.44^{bA}	102.22 ± 8.07^{aA}
(IC_{50} µg/ml)	Sun	122.57 ± 18.36^{aA}	195.48 ± 5.22^{bA}	239.03 ± 13.37^{cC}
	Parabolic dome	147.91 ± 9.96^{aB}	236.99 ± 16.57^{bB}	135.18 ± 5.48^{aB}
TPC	Oven	66.04 ± 5.12^{aA}	49.66 ± 2.85^{bA}	42.35 ± 5.48^{bA}
(µg GAE/mg of extract)	Sun	31.53 ± 1.52^{aB}	33.87 ± 5.08^{aB}	26.86 ± 2.16^{bB}
	Parabolic dome	37.97 ± 1.77^{aC}	36.07 ± 1.26^{aB}	38.11 ± 3.31^{aA}
TFC	Oven	47.41 ± 1.93^{aA}	13.37 ± 1.37^{bA}	5.48 ± 0.98^{cA}
(µg QE/ mg of extract)	Sun	33.19 ± 7.69^{aB}	9.19 ± 1.65^{bB}	6.39 ± 1.48^{cA}
	Parabolic dome	40.53 ± 8.77^{aAB}	12.57 ± 2.42^{bA}	4.71 ± 0.96^{cA}

Ascorbic acid was used as a reference standard with IC_{50} of DPPH $= 2.41 \pm 0.11$ µg/ml and IC_{50} of ABTS $= 2.78 \pm 0.03$ µg/ml.
Different superscripts, $^{a–c}$ within a row, $^{A–C}$ within a column, are significantly different ($p < 0.05$) using Duncan's multiple range test.

Effects of ethanolic extracts of *C. nutans* on LPS-induced NO production in RAW 264.7 cells

NO production, a key inflammatory mediator, was measured to assess the anti-inflammatory activity of the extracts. LPS markedly increased NO levels compared to the control, whereas treatment with the 95%, 70%, and 50% ethanolic extracts significantly reduced NO production in a dose-dependent manner at 62.5–500 µg/ml (Fig. S1). The IC_{50} values were 53.13 ± 10.79 µg/ml for the 95% extract, 89.05 ± 7.41 µg/ml for the 70% extract, and 118.23 ± 14.16 µg/ml for the 50% extract.

HPLC chromatograms of *C. nutans* ethanolic extracts

The chromatograms at 330 nm revealed four major peaks within 35 min, with vitexin eluting at 24.49 min and a prominent unidentified compound (compound X) at 20.98 min (Fig. S2a–d). Although distinct from schaftoside, compound X shared a nearly identical UV spectrum (Fig. S2a) and was also present in the schaftoside reference standard (Fig. S3). Previous report showed that isoschaftoside elutes immediately after schaftoside using HPLC system similar to ours [35].

Flavonoids typically exhibit two UV absorption bands—Band I (320–385 nm, B-ring) and Band II (250–285 nm, A-ring) [35]. Compound X showed Band I at 335.4 nm and Band II at 271.2 nm (Fig. S2a), matching the spectra of compound X in the schaftoside standard (335.6 and 271.3 nm) (Fig. S3) and schaftoside itself (335.2 and 271.1 nm) (Fig. S2a). These spectral similarities, together with reported elution patterns, strongly suggest that compound X is isoschaftoside. A study in microglial cells demonstrated that isoschaftoside effectively inhibited LPS-induced NO production and reduced iNOS, TNF-α, IL-1β, and COX-2 expression, showing activity even at 10 µM and optimal effects at 200 µM [36]. Thus, the presence of compound X as the major component becomes noteworthy, particularly if it is confirmed to be isoschaftoside.

Additionally, schaftoside—a major flavonoid in *C. nutans* from Chiang Mai [37]—was absent in our Suphanburi samples, indicating geographic variation in flavonoid profiles.

Table 3 Intra-day and inter-day precision, recovery and accuracy data.

Standard	Concentration	Precision %RSD		Amount added	% Recovery	
	(µg/ml)	Intra-day	Inter-day	(µg/ml)	Recovery (%)	RSD (%)
	30	0.541	1.279	30	101.709	0.750
Vitexin	15	0.655	0.518	15	107.817	1.081
	7.5	0.185	0.386	7.5	102.368	0.884

Table 4 Effect of drying methods and extraction solvents on vitexin content in *C. nutans*.

Drying method	Vitexin (µg/g dry weight)		
	95% Ethanol	70% Ethanol	50% Ethanol
Oven	308.67 ± 1.36^{aA}	12.79 ± 0.50^{bA}	3.86 ± 0.33^{cB}
Parabolic dome	163.17 ± 10.35^{aB}	6.17 ± 1.37^{bB}	6.10 ± 0.38^{bA}
Sun	0.34 ± 0.00^{aC}	not detected	not detected

Different superscripts, $^{a–c}$ within a row, $^{A–C}$ within a column, are significantly different ($p < 0.05$) using Duncan's multiple range test.

Validation of the HPLC method for vitexin quantification

Method validation was performed by evaluating linearity, LOD, LOQ, specificity, accuracy, and precision. Vitexin showed excellent linearity ($R^2 = 0.9996$), meeting ICH criteria (>0.995) (Fig. S4). The LOD and LOQ were 0.178 and 0.593 µg/ml, respectively. Intra- and inter-day precision values (%RSD) complied with ICH requirements (Table 3). Recovery ranged from 101.71% to 107.82%, within the acceptable 90–115% range. These results confirm that the developed HPLC method is suitable for quality control of vitexin in *C. nutans* leaf material.

Vitexin content in *C. nutans* extracts

Using the validated method, the 95% ethanolic extract showed the highest vitexin content (308.67 ± 1.36 µg/g dry weight), significantly higher than the 70% and 50% extracts. Oven-dried leaves yielded the greatest vitexin levels, whereas sun-dried samples showed minimal or undetectable vitexin in the 70% and 50% ethanolic extracts and only trace amounts in the 95% extract (Table 4).

Furthermore, it has been reported that the use of various plant growth regulators in the tissue culture of *C. nutans* can enhance the levels of bioactive compounds, including total phenolics, flavonoids, and phytosterols, as well as antioxidant activity. Therefore, this technique may have potential for developing high-vitexin lines for subsequent propagation [38].

CONCLUSION

This study provides a complete procedure for preparing *C. nutans* leaf extracts for anti-inflammatory evaluation—from optimized drying and solvent extraction to quantification using a validated HPLC-DAD method capable of clearly resolving the major flavonoids, including schaftoside, isoschaftoside (compound X), orientin, and vitexin. Future work should focus on selecting *C. nutans* plants with high vitexin content, as vitexin exhibits stronger biological activity than other apigenin-C-glycosides. Schaftoside was absent in the Suphanburi sample, whereas compound X, likely isoschaftoside, was the predominant constituent; its identity requires further confirmation. Improvements to the drying process—particularly by equipping the parabolic dome with temperature and humidity control—may offer more efficient and cost-effective drying.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2026.042.

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Appendix A. Supplementary data

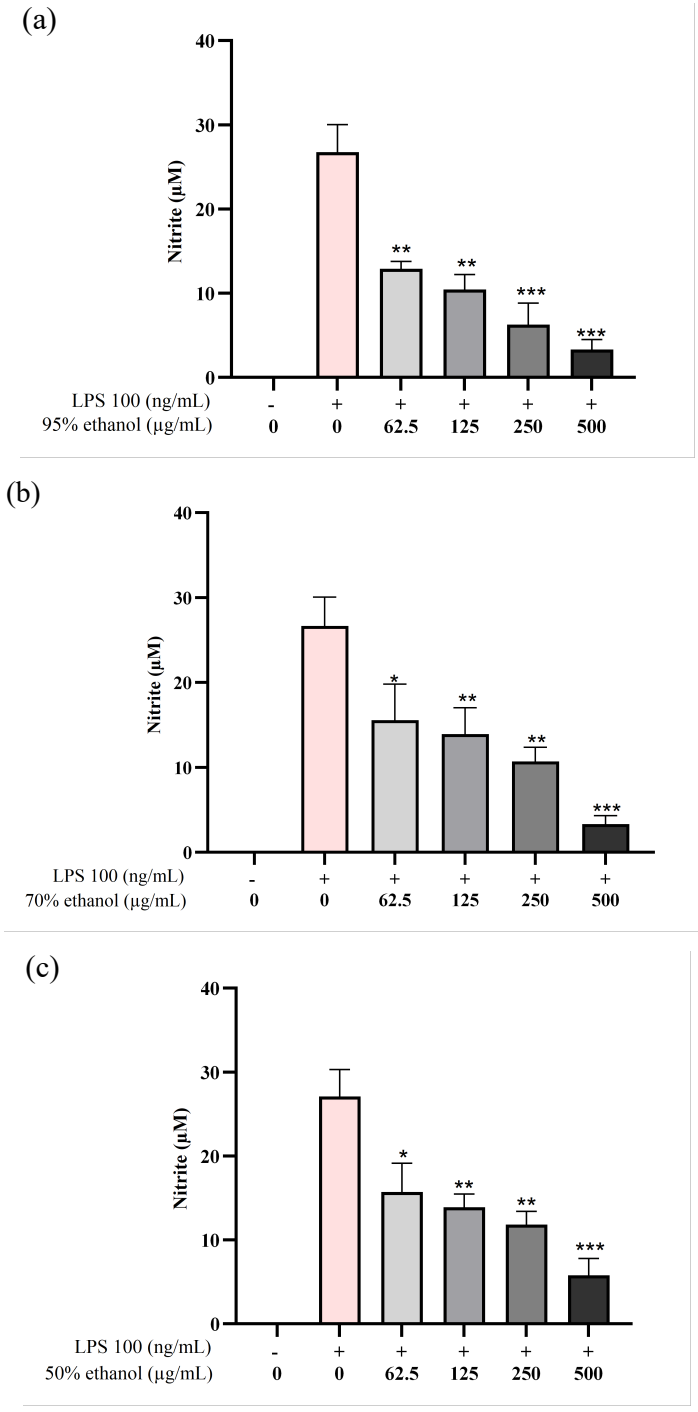

Fig. S1 Effects on NO Production in LPS-stimulated RAW 264.7 cells following exposure to various concentrations of 95% ethanolic extracts (a), 70% ethanolic extracts (b), and 50% ethanolic extracts (c). Values represent the mean ± SD of three independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p \leqslant 0.001$, compared with the LPS group.

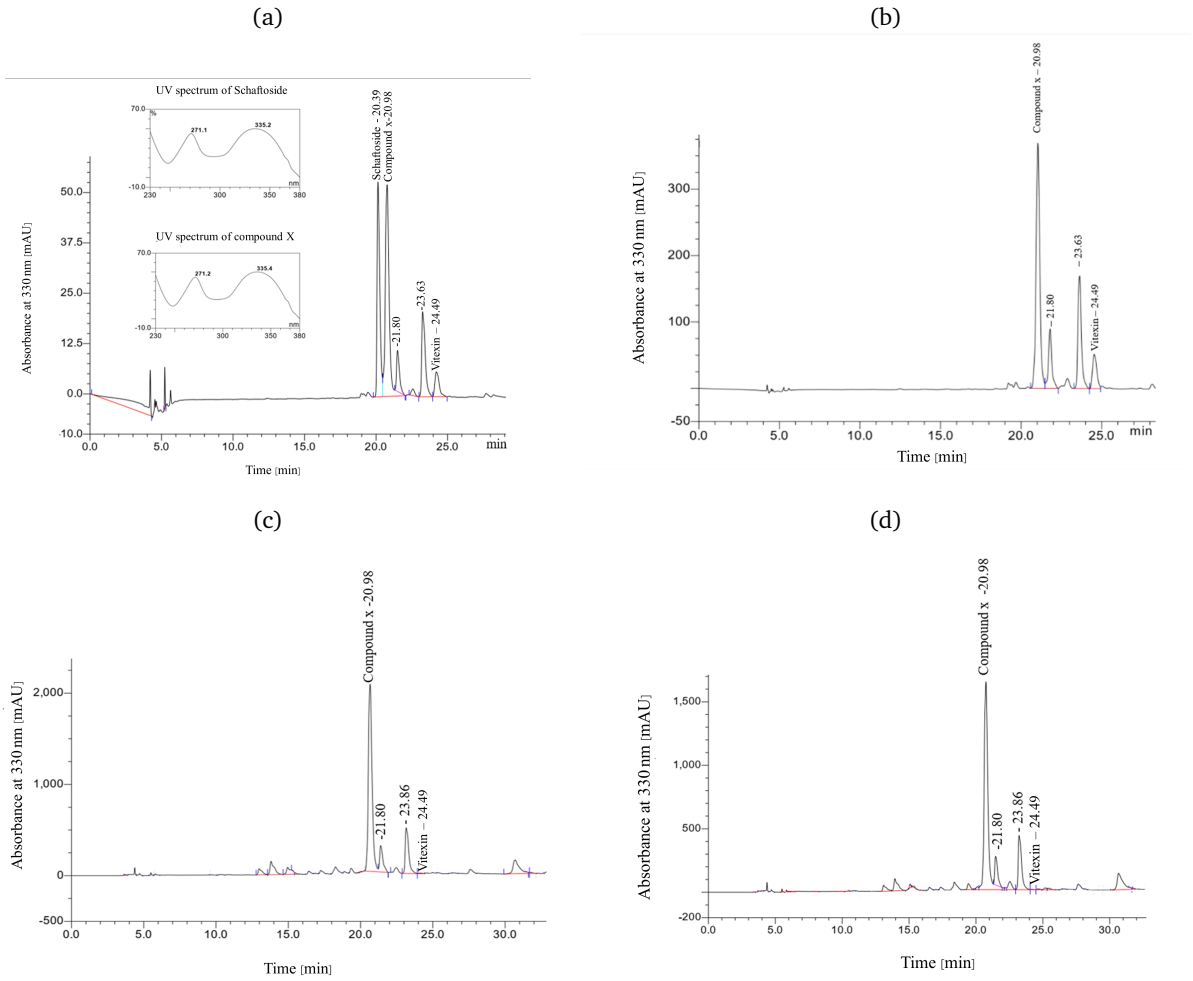

Fig. S2 HPLC chromatograms of *C. nutans* 95% ethanolic extract spiked with Schaftoside (30µg/ml) (a), *C. nutans* 95% ethanolic extract (b), *C. nutans* 70% ethanolic extract (c), *C. nutans* crude 50% ethanolic extract (d).

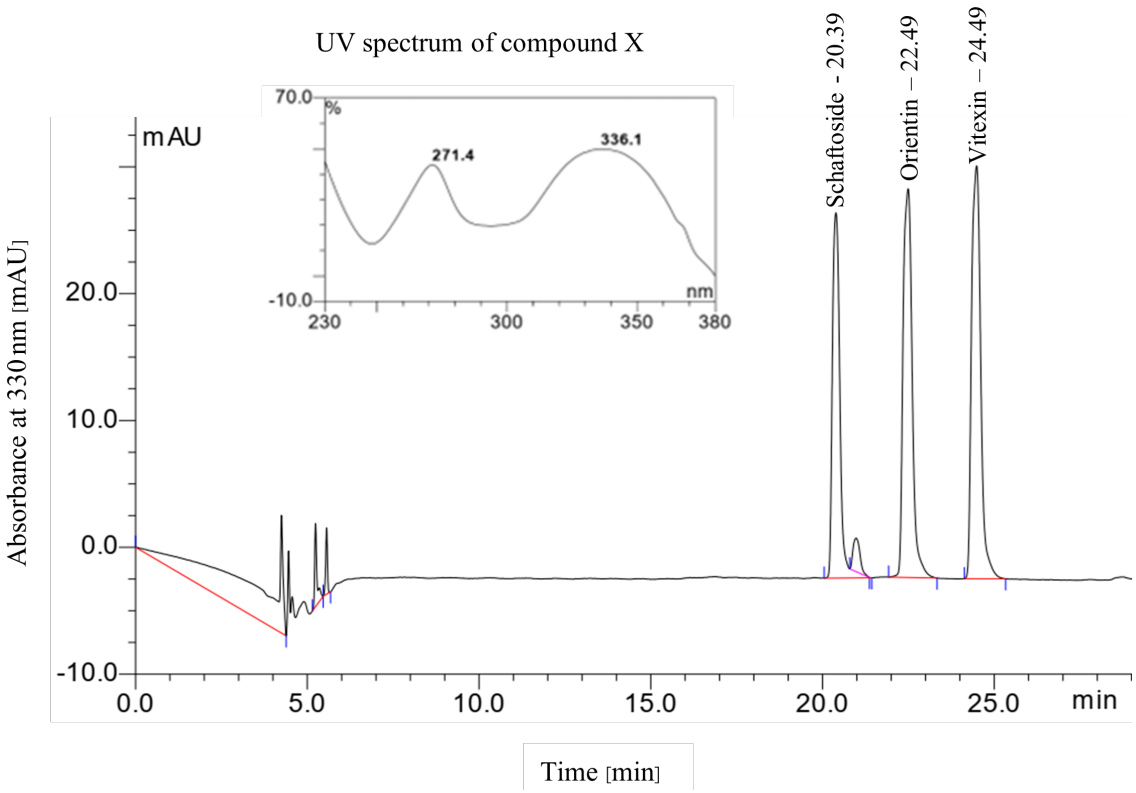

Fig. S3 HPLC chromatogram of mixed standards; Schaftoside, Orientin, and Vitexin.

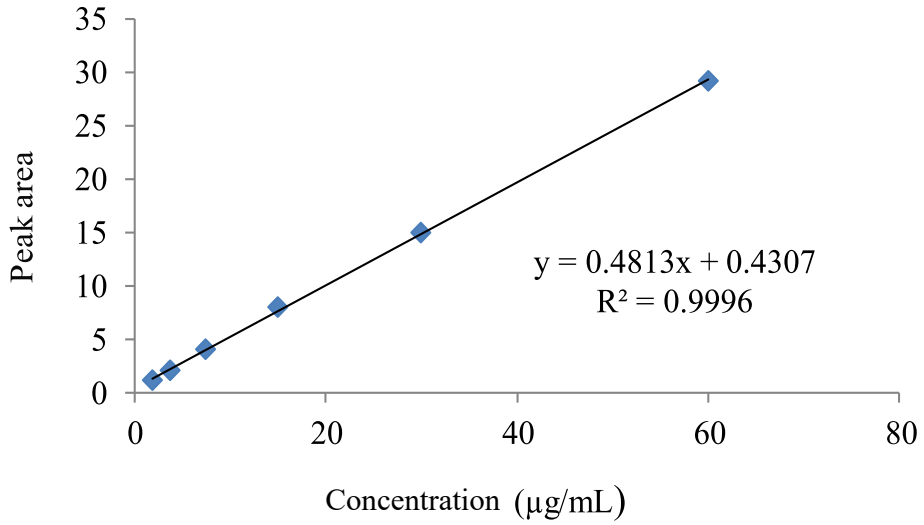

Fig. S4 Calibration curve for standard Vitexin.