BMC Complementary Medicine and Therapies

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Anti-inflammatory activity of *Curcuma wanenlueanga* Saensouk, Thomudtha & Boonma rhizomes and the search for its bioactive markers by harmonizing bioassay-guided isolation and network pharmacology

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Abstract

Background Rhizomes of *Curcuma wanenlueanga* Saensouk, Thomudtha & Boonma have been used in Thai traditional medicine and are included as an ingredient in a Thai traditional liniment formula listed by the Ministry of Public Health of Thailand for treating symptoms related to joints and muscle inflammation. However, anti-inflammatory activity and bioactive constituents of *C. wanenlueanga* have not yet been investigated. Thus, this study aimed to investigate the anti-inflammatory activity and underlying mechanism of *C. wanenlueanga* rhizome extract and its responsible bioactive components.

Methods The fractionation of bioactive compounds from *C. wanenlueanga* extract was guided by antioxidant activity on DPPH and Griess assays, and anti-inflammatory activity on LPS-induced RAW 264.7 cells. The biological activities of isolated compounds were first predicted by network pharmacology and further confirmed in cell-based assay with LPS-induced RAW 264.7 cells and enzyme-linked immunosorbent assay (ELISA) of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6).

Results The ethanolic extract of *C. wanenlueanga* rhizomes was proved to show anti-inflammatory activity on LPS-induced RAW 264.7 cells. Three curcuminoids including curcumin (**1**), demethoxycurcumin (**2**), dihydrodemethoxycurcumin (**3**), and two sesquiterpenoids, namely curcumenone (**4**), and zedoarondiol (**5**) were separated from anti-inflammatory fractions of *C. wanenlueanga* extract. The anti-inflammatory activity of these compounds to attenuate the productions of TNF-α and IL-6 was predicted by pharmacological network. The inflammatory assays, including the Griess assay for NO and ELISA for TNF-α and IL-6, confirmed that all isolated compounds reduced the production of these inflammatory mediators.

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Conclusion The present study shows the accordance between the results from pharmacological network and cell-based assays, which indicate the anti-inflammatory activity of *C. wanenlueanga* rhizomes and their bioactive constituents. This suggests the potential of bioactive compounds **1–5** to be used for quality assessment of *C. wanenlueanga* extract in the development of herbal products.

Keywords *Curcuma wanenlueanga*, Zingiberaceae, Anti-inflammatory activity, Bioassay-guided isolation, Network pharmacology, Bioactive marker

Background

Inflammation is a defense mechanism of the human immune system in response to irritants, typically leading to heat, swelling and redness of the affected area [1]. Acute inflammation occurs after injury and normally lasts for a few days to a week. If it does not resolve in time, it will progress to chronic inflammation in around six weeks, leading to the tissue damage and change in tissue integrity [2]. In an inflammation process, inflammatory cells such as neutrophils and macrophages migrate to the site of inflammation [3], with a secretion of key cytokines including interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) [4]. Inflammation can occur in every part of the body, and some related conditions, such as diabetes, cardiovascular disease and allergies, can be life-threatening. Additionally, muscle and joint inflammatory diseases, including myositis, gout, and rheumatoid arthritis (RA), cause discomfort, severe pain, permanent loss of functions, and critically affect all aspects of patient's quality of life. Several approaches are applied to treat muscle and joint inflammatory diseases, such as physical therapy, medication and surgery [5]. The first method is safe but takes a long time and is sometimes not medically effective. Surgery is an extreme method to deal with terminal conditions. Though, this method can lead to irreversible adverse effects. Nonsteroidal anti-inflammatory drugs (NSAIDs) are effective drugs that reduce pain, inflammation, and fever. Nevertheless, they have some potential adverse effects, including ulcers and bleeding in the gastrointestinal tract [6], elevated blood pressure [7], risk of heart attack [8], kidney and liver damages [9].

Recently, herbal medicine has gained attention as an alternative way to alleviate muscle and joint inflammation. Several medicinal plants in the *curcuma* genus, such as turmeric (*Curcuma longa* L.) [10], *Curcuma aromatica* Salisb [11]. and *Curcuma latifolia* Roscoe [12], have been proved to relieve inflammation. As the growing use of herbal medicine for treatment of various diseases [13], studies on medicinal plants to deal with inflammatory diseases remain an interesting area of research worldwide. Providentially, network pharmacology has been established to explain the relationship among chemical constituents in plants, drug targets, signaling pathways and diseases in holistic way [14]. For instance, Shi and colleagues explored the anti-inflammatory and antioxidant constituents of *Solanum melongena* L. and investigated their mechanisms using network pharmacology [15]. Limcharoen and coworkers utilized network pharmacology to disclose the ability of (–)-dendroparishiol from *Dendrobium parishii* to manage bacterial meningitis-associated neuroinflammation [16]. These support the potential of network pharmacology, which helps scientists open a new possibility to discover useful medicinal herbs and their bioactive constituents.

Curcuma wanenlueanga Saensouk, Thomudtha & Boonma (Fig. 1A) is a plant species in the Zingiberaceae family. It distributes and is cultivated through northern part of Thailand. This perennial herb could be up to 150 centimeters tall and has ovoid rhizome with internal yellow with darker core. The specific epithet of this plant was derived from its Thai vernacular name "Wanen-lueang", which mean "yellow herb related to tendons" [17]. In Thailand, *C. wanenlueanga* rhizomes have been recognized in several documents for their traditional use (Fig. 1B), including treatment of beriberi related to tendon, paralysis, sprains, bruise, swelling, backache, joint pain and tendonitis [18]. *C. wanenlueanga* is also listed as an ingredient in a Thai traditional liniment formula certified by the Ministry of Public Health of Thailand [19]. There is a previous study revealed antioxidant, anti α-glucosidase activities *C. wanenlueanga* [20]. However, preliminary investigation of anti-inflammatory activity and chemical constituents that are responsible for the bioactivity of *C. wanenlueanga* has not been investigated. Thus, this study aimed to investigate the anti-inflammatory activity of *C. wanenlueanga* rhizomes. Bioassay-guided fractionation was used to obtain bioactive constituents. The targets and mechanisms of action of isolated compounds were then forecasted by network pharmacology and confirmed by enzyme-linked immunosorbent assay (ELISA).

Methods

Chemicals

Silica gel (SiO_2) of 230–400 and 70–230 mesh size (cat. no. 1.09385 and 1.07734), SiO_2 thin-layer chromatography (TLC) plate (cat. no. 1.05554), SiO_2 high performance thin-layer chromatography (HPTLC) plate (cat. no. 1.05548), chloroform-d1 deuterated solvent (cat. no. 1.02446), analytical grade dimethyl sulfoxide (DMSO,

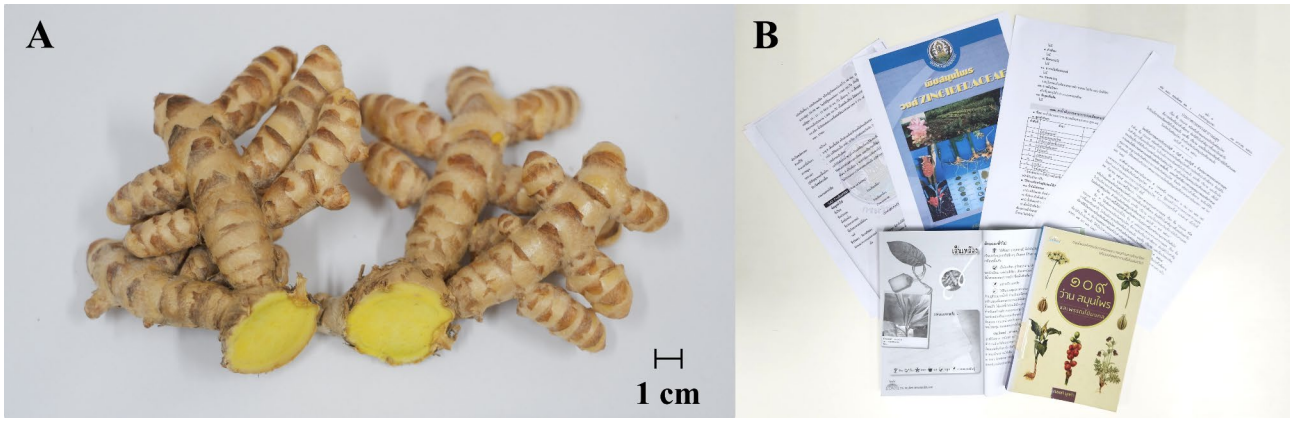

Fig. 1 *Curcuma wanenlueanga* Saensouk, Thomudtha & Boonma and its ethnobotanical references. (**A**) Morphological features of a fresh rhizome of *Curcuma wanenlueanga* and (**B**) its medicinal descriptions in numerous references including, notification of the Ministry of Public Health, *Herbs in Zingiberaceae*, and *109 Herbs and Auspicious Plants*

cat. no. 1.02952), and absolute ethanol (EtOH, cat. no. 1.00983) were acquired from Merck KGaA (Darmstadt, Germany). Sephadex LH-20 resin (cat. no. 17009001) was garnered from Cytiva (Marlborough, Massachusetts, USA). Analytical grade methanol (MeOH, cat. no. MACR3016-68) was acquired from Avantor (Radnor, Pennsylvania, USA). Reagent grade n-butanol (n-BuOH, cat. no. KA107) was garnered from Kemaus (Cherrybrook, New South Wales, Australia). Commercial grade hexanes, dichloromethane (DCM), ethyl acetate (EtOAc), acetone and methanol (MeOH) were purchased from T.S. Interlab LP, Bangkok, Thailand, and were distilled before use. Sodium nitroprusside (SNP, cat. no. 05958) was acquired from Loba Chemie PVT. LTD. (Mumbai, India). Phosphate buffer saline (PBS, cat. no. 10010023) was garnered from Gibco (Thermo Fisher Scientific, Massachusetts, USA). RAW 264.7 macrophage cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM, cat. no. 1TFS-1CC-12100046), Griess reagent (cat. no. G4410), 2,2-Diphenyl-1-picrylhydrazyl (DPPH, cat. no. D9132), lipopolysaccharides (LPS, cat. no. L2630-100MG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, cat. no. 475989) and penicillin-streptomycin solution (cat. no. P4333-100ML) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS, cat. no. P30-3031) was purchased from PAN-Biotech GmbH (Aidenbach, Germany). ELISA kits for TNF-α and IL-6 (cat. no. 430901 and 431304) were garnered from BioLegend (San Diego, CA).

Plant material

C. wanenlueanga rhizomes were collected from Uttaradit Province, Thailand, under legal and permitted conditions. The plant sample was identified by Associate Professor Thatree Phadungcharoen, the taxonomist at Faculty of Pharmaceutical Sciences, Chulalongkorn University. A voucher specimen (SS-894) was deposited at the Museum of Natural Medicines, Chulalongkorn University, Bangkok, Thailand.

Extraction and bioassay-guided isolation of *C. wanenlueanga*

To obtain a crude extract, the fresh rhizomes of *C. wanenlueanga* were cleaned, sliced, dried in a hot air oven at 50 °C for 24 h and successively ground into powder. The powder was then subjected to ultrasonic-assisted extraction. Briefly, 1.5 kg of *C. wanenlueanga* powder was added into a stainless-steel pot with 9 L of 70% EtOH. The pots were placed in GT SONIC D27 ultrasonic cleaner (GuangDong GT Ultrasonic Co., Ltd, Shenzhen, China) and sonicated at 30 °C for 1 h. The extracts were successively collected and filtered through cotton wool. The rhizomes were re-extracted until exhausted. The filtrate was pooled together and successively evaporated under vacuum to get *C. wanenlueanga* rhizome ethanolic extract.

C. wanenlueanga rhizome ethanolic extract was redissolved in water and subjected to liquid-liquid extraction with EtOAc, *n*-BuOH to get EtOAc, BuOH and H_2O partitions. The partitions were screened for antioxidant and anti-inflammatory activities. The most active partition was further separated by vacuum liquid chromatography (VLC), eluted with hexanes-dichloromethane and MeOH-dichloromethane gradient systems. The obtained fractions were combined based on their TLC patterns. Fractions with desired biological activities were then selected and successively separated using a series of SiO_2 and Sephadex LH-20 columns to obtain pure compounds. The isolated compounds were analyzed on a Bruker Ascend 400 NMR spectrometer (Massachusetts, USA) to acquire ^{1}H-NMR and ^{13}C-NMR spectra, and the

molecular weight were determined by JEOL AccuTOF™ LC series DART™ (Tokyo, Japan).

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assay

The DPPH antioxidant assay used to determine the antioxidant capacity of the crude extract, partitions, and fractions of *C. wanenlueanga* was conducted with some modifications [21]. Briefly, 1 mg each of *C. wanenlueanga* crude extract, partitions, and fractions was dissolved in 1 mL of MeOH to obtain sample solutions of 1 mg/mL. Then, 20 µL of each sample solution was mixed with a 180 µL of the 150 µM DPPH solution in a 96-well plate. The microplate was incubated for 30 min in the dark at room temperature and then analyzed using CLARIOstar® Plus microplate reader (BMG Labtech, Ortenberg, Germany) at 515 nm. MeOH was used as a blank, while Trolox and ascorbic acid were used as positive controls. The DPPH radical scavenging activity was determined using the following formula:

$$\text{DPPH radical scavenging activity}(\%) = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

where A_c is the absorbance of DPPH without sample, and A_s is the absorbance of the sample mixed with DPPH solution. The assay was performed in triplicate.

Cell-free Griess assay

The scavenging activity of the extract, partitions, and fractions of *C. wanenlueanga* against nitric oxide produced from sodium nitroprusside (SNP) was determined by Griess assay with some modification [22]. Briefly, *C. wanenlueanga* crude extract, partitions, and fractions were separately dissolved in 35% DMSO to obtain 200 µg/mL sample solutions. Then, 50 µL of each sample was mixed with an equal volume of 20 mM SNP in a 96-well plate. The mixtures were incubated at 25 °C, under an ambient light for 90 min, then 100 µL of Griess reagent was added, and then analyzed using CLARIOstar® Plus microplate reader at 546 nm. Reaction mixtures without Griess reagent were used as blanks, while Trolox was used as a positive control. The NO scavenging activity was calculated in the same way as for the DPPH assay.

Cell culture and cell viability assay

RAW 264.7 macrophage cells (ATCC) were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and maintained in a CO_2 incubator at 5% CO_2 and 37 °C.

Cell viability assay was performed using MTT assay, which is based on the ability of dehydrogenase enzymes in the cells to convert MTT to purple formazan granules. The cells were seeded into 96-well plates at a density of 20,000 cells/well for 24 h. The cells were then treated with various concentrations of the extract, partitions, fractions (0, 12.5, 25, 50, 100, and 200 µg/mL), or isolated compounds (0, 0.25, 0.5, 1, 2, 4, 8 and 16 µM) for 24 h. After treatment, the media was removed, followed by addition of MTT solution and incubated for 3 h. The crystal formazan was dissolved using DMSO. The absorbance was further assessed using a microplate reader at 570 nm. The cell viability assay was performed first to obtain a maximum non-toxic concentration (MNTC), which was then used in the NO and ELISA assays.

Evaluation of anti-inflammatory activity on RAW 264.7 cells

Cell-based Griess assay was performed to indicate the presence of the inflammatory mediator, NO, by measuring the nitrite level in the media [12]. Briefly, the cells were seeded in 48-well plates at a density of 100,000 cells/well for 24 h. The cells were then pretreated with the MNTC of the extract, partitions and compounds for 1 h. After pretreatment, the cells were incubated with LPS at a concentration of 100 ng/mL for 12 h. Then, 100 µL of the treated media was added to a 96-wells plate, followed by addition of 100 µL Griess reagent and incubated for 10 min. The absorbance was measured under microplate readers at 520 nm. Griess reagent was prepared according to the protocol provided by the manufacturer (Sigma Aldrich).

ELISA test

The expression levels of proinflammatory cytokines, including TNF-α and IL-6, in LPS-stimulated RAW 264.7 cells were determined using ELISA kits (BioLegend, San Diego, CA) [12]. The concentrations of cytokines in the culture supernatant were determined using their respective standard curves.

Pharmacological network

An overview of the interactions between compounds isolated from *C. wanenlueanga* and the biological targets related to a disease was predicted by network pharmacology. The SMILES strings and SDF files of isolated compounds were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/), and then used in Swiss Target Prediction (http://www.swisstargetprediction.ch/), Similarity ensemble approach (SEA) (https://sea16.docking.org/) and PharmMapper (https://www.lilab-ecust.cn/pharmmapper/) databases to obtain the potential targets. The targets from the databases were pooled, and duplicates were removed. Since rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory disease, it was used as a suitable keyword for identifying human inflammation-related target genes in the databases, including the DisGeNET database (fit score ≥ 0.1) (https://www.disgenet.org/), the OMIM® database (https://www.omim.org/) and the GeneCards database(score ≥ 10) (https://w

ww.genecards.org/). The target genes curated from each database were combined, and duplicates were removed. All gene targets obtained from all databases were validated and standardized using UniProt database (https://www.uniprot.org/). The overlap between the targets of each compound, their combinations, and RA-related targets was evaluated using Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). The overlapping gene targets were inputted to STRING database (https://string-db.org/) to obtain protein-protein interaction (PPI) network. The PPI network was transferred to Cytoscape software (v3.10.0), and cytoHubba plug-in (v0.1) was employed to identify the top 10 genes with the highest degree of interactions. Moreover, the potential mechanisms of actions and pathways involved with gene products were described by using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. GO and KEGG analysis were performed using an online tool for bioinformatic data analysis (http://www.bioinformatics.com.cn/).

Statistical analysis

All results are presented as the mean ± SD. The results from the biological assays were analyzed by GraphPad Prism 9 software using one-way analysis of variance (one-way ANOVA), followed by Dunnett, Bonferroni, or Tukey post hoc tests. A P value < 0.05 was considered statistically significant.

Results

The ethanolic extract of *C. wanenlueanga* rhizomes possessed antioxidant and anti-inflammation activities

Ultrasonic-assisted extraction was utilized to obtain crude *C. wanenlueanga* extract with 15.7% extraction yield. The extract showed 34.3 ± 5.8% and 36.5 ± 1.6% scavenging activity in the DPPH and NO assays, respectively (Fig. 2). To perform an anti-inflammatory test on LPS-induced RAW 264.7 cells, the cell viability was first assessed using the MTT assay to obtain a maximum non-toxic concentration (MNTC) of the extract. The MNTC of the *C. wanenlueanga* crude extract was determined to be 50 μg/mL (92.8 ± 6.3% cell viability) (Fig. S1), and the extract showed anti-inflammatory activity on RAW 264.7 cells with an IC_{50} value of 13.33 ± 5.51 μg/mL (Table 1).

Three curcuminoids and two sesquiterpenoids were obtained by bioassay-guided isolation

To obtain anti-inflammatory constituents, *C. wanenlueanga* extract was separated into EtOAc, BuOH, and H_2O partitions (Fig. 2). The antioxidant activities of the three partitions were assessed using DPPH and cell-free Griess assays. The H_2O partition was the least active partition with no DPPH scavenging activity and 11.0 ± 1.4% NO scavenging activity. In contrast, the EtOAc partition showed the highest antioxidant activities with 45.0 ± 0.6% DPPH scavenging and 41.1 ± 0.4% NO scavenging activities. Additionally, the EtOAc partition was proved to show MNTC of 25 μg/mL (Fig. S1) and displayed potent anti-inflammatory activity on RAW 264.7 cells with the IC_{50} value of 5.4 μg/mL, which is lower than that of the crude extract (Table 1). Further fractionation of the EtOAc partition with VLC resulted in 4 fractions, labeled A_1-A_4 (Fig. 2). Fractions A_3 and A_4, which possessed more than 30% scavenging activities on DPPH and NO assays, were subsequently evaluated for their anti-inflammatory activity on RAW 264.7 cells. The MNTC of fractions A_3, determined by MTT assay, was 12.5 μg/mL, while fraction A_4 had a higher MNTC of 25 μg/mL (Fig. S1). Fractions A_3 and A_4 exhibited anti-inflammatory activity with the IC_{50} values of 0.91 ± 0.03 and 3.15 ± 0.74 μg/mL, respectively, which are significantly lower than that of crude extract, but not significantly different from that of the positive control (dexamethasone) (Table 1). According to the results, fractions A_3 and A_4 were further explored for bioactive compounds. Fraction A_3 (4.6 g) was subjected to SiO_2 (230-400 mesh) column chromatography, eluting with 20% EtOAc/hexanes to yield eight fractions (B_1-B_7). Fraction B_7 (285 mg) was found to be curcumin (**1**). Fraction B_4 (645 mg) was continued on SiO_2 column chromatography, eluting with 10% acetone in hexanes to obtain fractions C_1-C_4. Fraction C_2 (449 mg) was separated through a Sephadex LH-20 column with MeOH to acquire fractions D_1-D_3. Fraction D_2 (300 mg) was separated by SiO_2 column chromatography eluting with 5% acetone/hexanes to obtain fractions E_1-E_5. Fraction E_2 (89.0 mg) was proved to be curcumenone (**4**). Fraction A_4 (27.9 g) was separated using a SiO_2 column, eluting with 25% acetone/hexanes to get fractions F_1-F_6. Zedoarondiol (**5**; 118 mg) was isolated from fraction F_3 (2.85 g) by using three consecutive SiO_2 columns, eluting with EtOH: CH_2Cl_2:hexanes (2:52:46), 10% acetone/hexanes, and 60% EtOAc/hexanes systems, respectively. Fraction F_4 (4.52 g) was subjected to a SiO_2 column, eluting with 40% EtOAc/hexanes to yield fractions J_1-J_5. Separation of fraction J_3 using a Sephadex LH-20 column led to the isolations of demethoxycurcumin (**2**; 268 mg) and dihyodrodemethoxycurcumin (**3**; 60.1 mg) (Fig. 2).

Compound **1** was obtained as a dark orange powder. The compound was determined to possess a molecular formular $C_{21}H_{20}O_6$ based on its high-resolution time-of-flight mass spectrometry (HRTOFMS) data at *m/z* 369.1169 (Calculated *m/z* 369.1338 for $[C_{21}H_{21}O_6]^+$) (Fig. S2). Compound **1** was proven to be curcumin by comparing ^1H and ^{13}C NMR spectra with those previously reported [23] (Fig. S3-S4 and Table S1).

Compound **2** was collected as an orange powder. The compound was determined to show a molecular formular

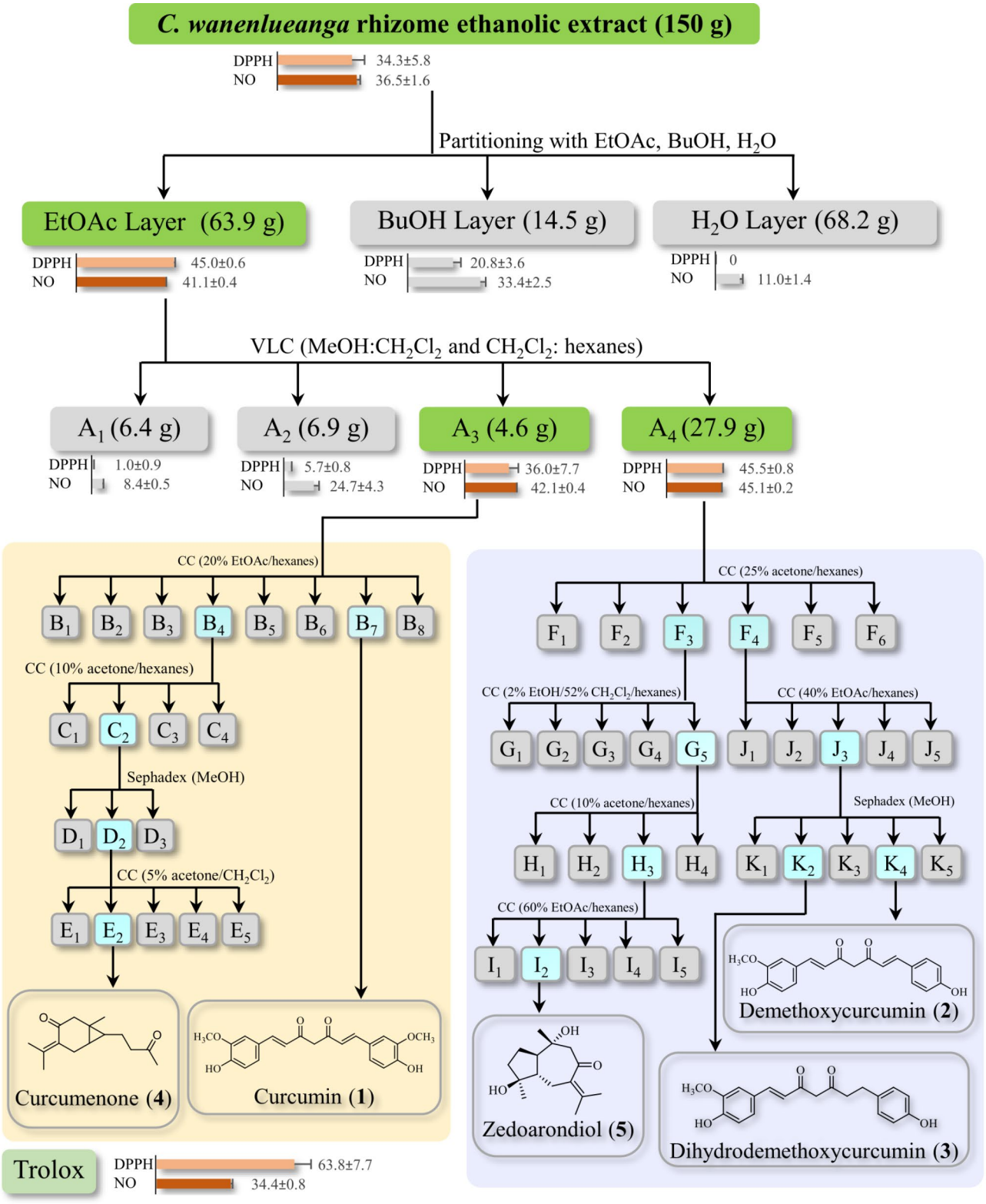

Fig. 2 Bioassay-guided fractionation of *C. wanenlueanga* extract. The bars under entries represent DPPH and nitric oxide (NO) scavenging activities. Samples were tested at a concentration of 100 µg/mL. Trolox at 10 and 20 µg/mL was used as a positive control in DPPH scavenging and NO scavenging assays, respectively. VLC = vacuum liquid chromatography; CC = column chromatography

Table 1 The IC_{50} of the extract, partition, and fractions of *C. wanenlueanga* on nitric oxide production in LPS-stimulated RAW 264.7 macrophage cells

Treatment	IC_{50} (μg/mL)
70% EtOH crude extract	13.33 ± 5.51^{b}
EtOAc partition	5.43 ± 0.65^{a}
Fraction A_3	0.91 ± 0.03^{a}
Fraction A_4	3.15 ± 0.74^{a}
Dexamethasone	3.08 ± 1.29^{a}

Dexamethasone was used as a positive control. The statistical differences were analyzed using one-way ANOVA followed by Tukey's test. Values with the same superscript letter within a column are not significantly different at $p < 0.05$

$C_{20}H_{18}O_5$ based on its HRTOFMS data at *m/z* 339.1163 (Calculated *m/z* 339.1232 for $[C_{20}H_{19}O_5]^+$) (Fig. S5). Compound **2** was elucidated to be demethoxycurcumin by comparing ^1H and ^{13}C NMR spectra with previously reported data [24] (Fig. S6-S7 and Table S2).

Compound **3** was isolated as a pale orange powder. The compound was determined to show a molecular formular $C_{20}H_{20}O_5$ based on its HRTOFMS data at *m/z* 341.1344 (Calculated *m/z* 341.1389 for $[C_{20}H_{21}O_5]^+$) (Fig. S8). Compound **3** was interpreted to be dihydrodemethoxycurcumin by comparing ^1H and ^{13}C NMR spectra with those previously reported [25] (Fig. S9-S10 and Table S3).

Compound **4** was acquired as yellow oil. The compound was determined to show a molecular formular $C_{15}H_{22}O_2$ based on its HRTOFMS data at *m/z* 235.1683 (Calculated *m/z* 235.1698 for $[C_{15}H_{23}O_2]^+$) (Fig. S11). Compound **4** was elucidated to be curcumenone by comparing ^1H and ^{13}C NMR spectra with previously reported data [26] (Fig. S12-S13 and Table S4).

Compound **5** was obtained as yellow oil. The compound was determined to show a molecular formular $C_{15}H_{24}O_3$ based on its HRTOFMS data at *m/z* 253.1805 (Calculated *m/z* 253.1804 for $[C_{15}H_{25}O_3]^+$) (Fig. S14). Compound **5** was identified to be zedoarondiol by comparing ^1H and ^{13}C NMR spectra with those previously reported [27] (Fig. S15-S16 and Table S5).

Bioactive compounds from *C. wanenlueanga* extract were predicted by network pharmacology to modulate inflammatory gene targets

The potential underlying mechanisms contributing to the bioactivity of the isolated compounds and their combination were investigated using network analysis. Data curated from databases showed 969, 934, 861 642, 394 and 1201 potential targets for curcumin, demethoxycurcumin, dihydrodemethoxycurcumin, curcumenone, zedoarondiol, and the combination of all compounds, respectively. In addition, 893 rheumatoid arthritis (RA)-related gene targets were retrieved from the databases (Fig. 3A). Venn diagrams revealed the number of overlapping genes between RA-related genes and the targets of the isolated compounds. The intersections were sorted in ascending order as 50, 71, 89, 100 and 104 genes for zedoarondiol, curcumenone, dihydrodemethoxycurcumin, curcumin, and dimethoxycurcumin, respectively. When considering the combined gene targets of all the compounds, a total of 123 intersecting genes with RA-related genes were identified (Fig. 3A and B). These findings demonstrated that compounds isolated from *C. wanenlueanga* are forecasted to target RA-related genes.

The intersecting genes between the isolated compounds of *C. wanenlueanga* and RA were further analyzed for their interactions using the STRING database, resulting in a protein-protein interaction (PPI) network (Fig. 4A). The network displayed complex interactions among the target proteins involved, comprising 123 nodes and 1810 edges. Among these, 120 nodes were interconnected with at least one other protein, while three protein targets exhibited no linkage. The PPI network was further analyzed using the CytoHubba plug-in to identify the top 10 ranked genes, including interleukin-6 (IL6), tumor necrosis factor (TNF), signal transducer and activator of transcription 3 (STAT3), matrix metalloproteinase-9 (MMP9), albumin (ALB), caspase-3 (CASP3), transcription factor Jun (JUN), B-cell lymphoma 2 (BCL2), epidermal growth factor receptor (EGFR), and hypoxia-inducible factor 1-alpha (HIF1A) (Fig. 4B). These key targets are predicted to play an important role in the effects of *C. wanenlueanga*, particularly its anti-inflammatory properties.

GO enrichment analysis was conducted to gain further insights into the biological mechanisms and pathways targeted by the chemical constituents of *C. wanenlueanga* associated with RA (Fig. 5A and D). The top five enriched biological processes included the regulation of inflammatory response, response to molecule of bacterial origin, response to lipopolysaccharide, response to oxidative stress, and regulation of cell-cell adhesion (Fig. 5A). The top five enriched cellular components were identified as membrane raft, membrane microdomain, membrane region, vesicle lumen, and secretory granule lumen (Fig. 5B). Molecular functions analysis revealed the top five enriched categories: protein tyrosine kinase activity, phosphate binding, protein phosphatase binding, endopeptidase activity, and non-membrane spanning protein tyrosine kinase activity (Fig. 5C).

KEGG pathway enrichment analysis highlighted the enrichment of top 5 pathways, including TNF signaling pathway, Kaposi sarcoma-associated herpesvirus infection, lipid, and atherosclerosis, PD-L1 expression and PD-1 checkpoint pathway in cancer, and C-type lectin receptor signaling pathway (Fig. 5D). These findings provide insight into potential mechanisms of action of *C. wanenlueanga*' s chemical constituents across various biological pathways.

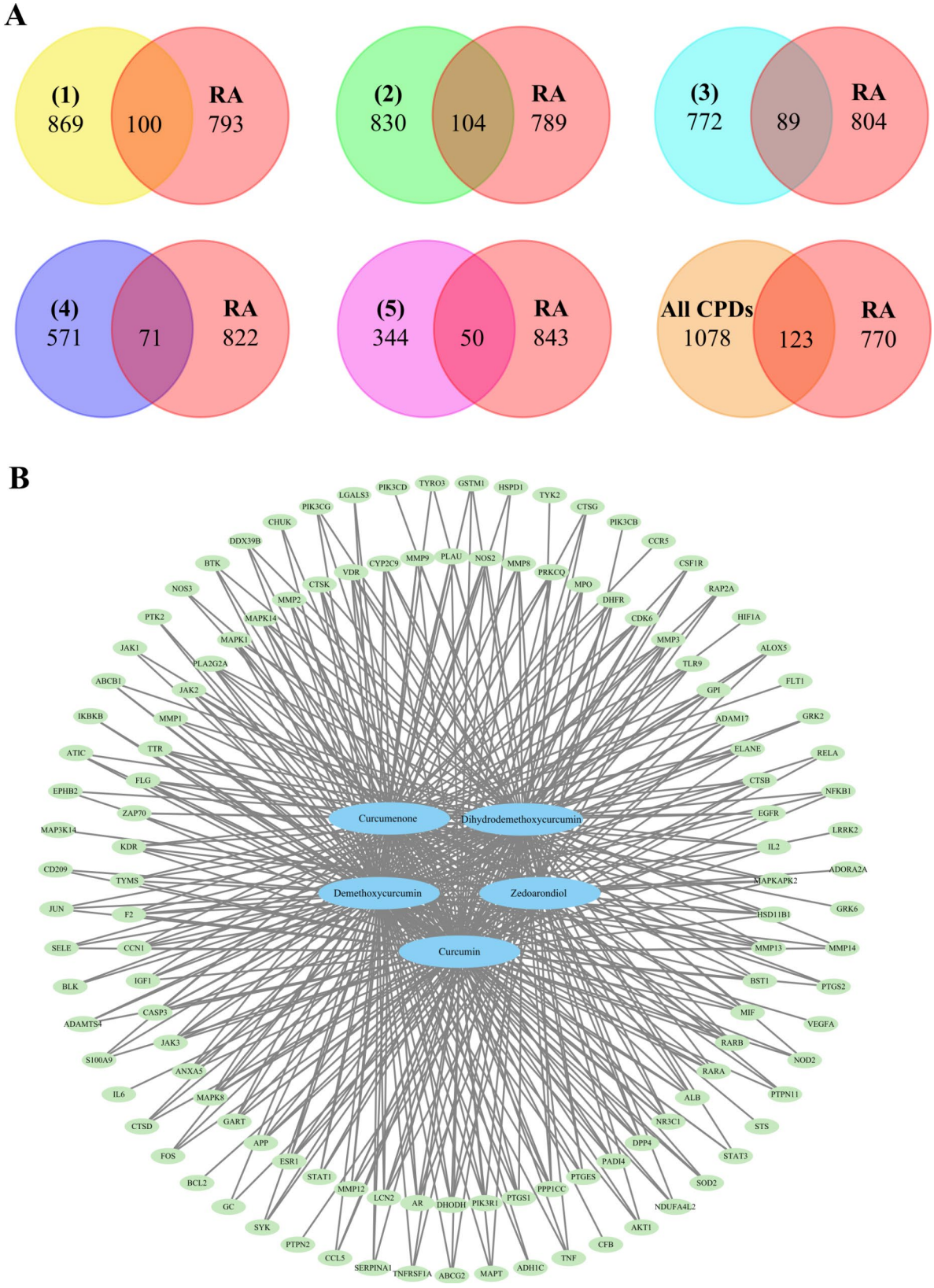

Fig. 3 Analysis of target genes of isolated compounds from *C. wanenlueanga* and genes expressed in inflammation. (**A**) Venn diagrams illustrated the overlapping between test compounds' target genes (curcumin (**1**), demethoxycurcumin (**2**), dihydrodemethoxycurcumin (**3**), curcumenone (**4**), zedoarondiol (**5**), and the combination of them) and rheumatoid arthritis-related genes (RA). (**B**) A network showing the interaction between isolated compounds from *C. wanenlueanga* extract and all potential targets for RA

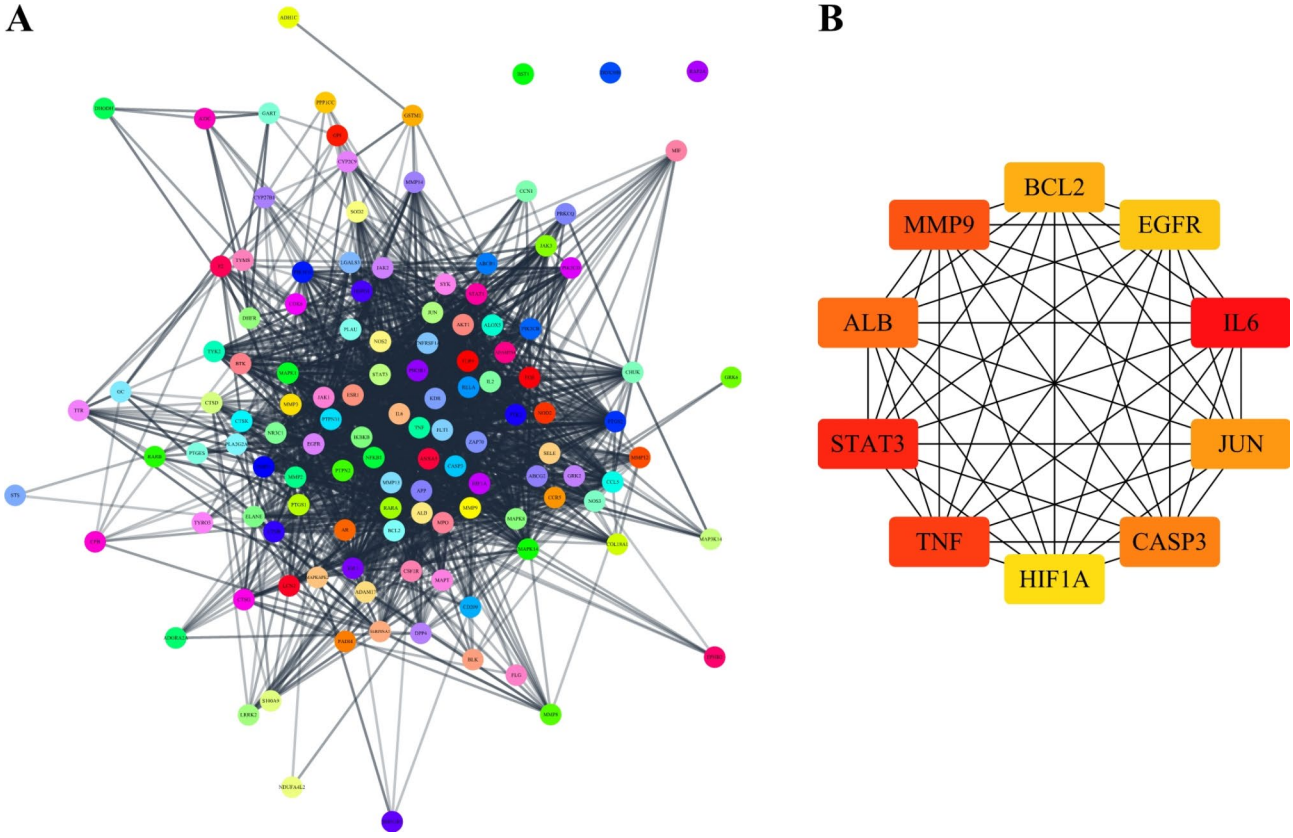

Fig. 4 Network analysis of isolated compounds from *C. wanenlueanga* extract and their interactions with RA-related genes. (**A**) Protein–protein interaction (PPI) network constructed using the STRING database, depicting interactions among 123 overlapping targets. (**B**) The top 10 core targets were identified using the cytoHubba plug-in, ranked by degree scores. The color gradient from red to yellow indicates decreasing degree of scores, with red indicating the highest-ranked targets

The TNF-α signaling pathway is a key pathway implicated in inflammation and is closely associated with RA. As shown in Fig. 6, the compounds isolated from *C. wanenlueanga* interact with multiple targets within the TNF-α signaling pathway. Various immune cells, including macrophages, express tumor necrosis factor receptor 1 (TNFR1), which responds to TNF-α and initiates inflammatory signaling. Upon activation, TNFR1 recruits adaptor proteins such as TNF receptor-associated factors (TRAF), receptor-interacting protein kinase 1 (RIP1), and TNFR1-associated death domain (TRADD). These adaptors mediate the activation of downstream inflammatory pathways, namely the NF-κB and mitogen-activated protein kinase (MAPK) pathways.

In the NF-κB pathway, TNFR1 activation stimulates NF-κB-inducing kinase (NIK), which phosphorylates and activates the IκB kinase (IKK) complex. The activated IKK complex subsequently phosphorylates the NF-κB inhibitor IκBα, leading to the release and activation of NF-κB. Once translocated to the nucleus, NF-κB drives the transcription of pro-inflammatory genes that perpetuate inflammation. In the MAPK pathway, transforming growth factor-β-activated kinase 1 (TAK1) is activated, triggering a signaling cascade to downstream MAPKs, including c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK). These MAPKs activate transcription factors such as activator protein 1 (AP-1), CCAAT-enhancer-binding proteins (c/EBPβ), and cAMP response element-binding protein (CREB), respectively. These transcription factors induce the expression of inflammatory cytokines, further amplifying the inflammatory response.

The findings suggest that the compounds isolated from *C. wanenlueanga* interact with multiple proteins in the TNF-α signaling pathway, including TNF, TNFR1, NIK, NF-κB, JNK, p38 MAPK, ERK, IKK, IL-6, and AP-1, thereby potentially attenuating RA-associated inflammation.

Bioactive compounds from *C. wanenlueanga* significantly reduced the concentration of nitric oxide, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in LPS-induced RAW264.7 cells

The anti-inflammatory effects of isolated compounds from *C. wanenlueanga* were confirmed by ELISA. From cytotoxicity test, compounds **4** and **5** had MNTC of 8

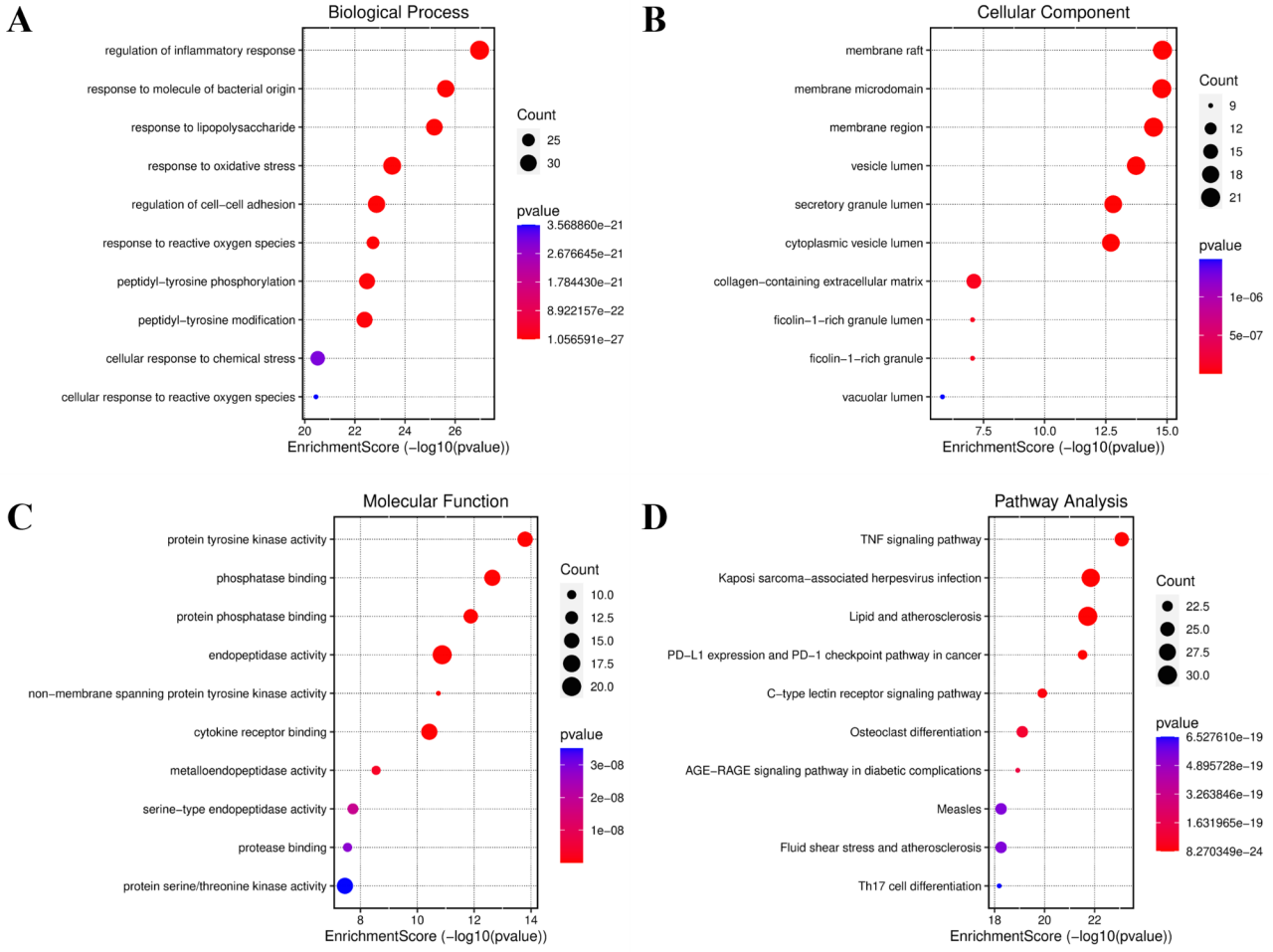

Fig. 5 Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of compounds isolated from *C. wanenlueanga*. Three plots of enriched ontologies represented biological processes (**A**), cellular components (**B**), and molecular functions (**C**), while the fourth plot displayed enrichment pathway analysis (**D**). In each plot term, the circle size represents the number of genes enriched, and the color represents the p-value

μM, while compounds **1** and **3** showed lower MNTC at 4 μM. Compound **2** possessed the lowest MNTC of 1 μM (Fig. S17). The nitrite level in the cell culture media was determined by Griess reaction. The results revealed that the nitrile level of LPS-induced RAW 264.7 cell significantly increased from approximately 1 μM to 18 μM (Fig. 7). Compound **1** at concentrations of 1 to 4 μM significantly decreased the nitrite level from 18.01 ± 1.30 μM to 3.74 ± 0.90 μM (Fig. 7A). Compound **2** significantly downregulated the level of nitrite from 16.96 ± 1.04 μM to 3.04 ± 1.24 μM with the doses of 0.25 to 1 μM (Fig. 7B). The nitrite level was significantly reduced from 17.46 ± 1.43 to 1.9 ± 0.83 μM as the concentration of **3** increased from 1 to 4 μM (Fig. 7C). Compounds **4** and **5** at the concentration of 8 μM significantly diminished the nitrite levels from 17.92 ± 1.08 to 8.98 ± 2.13 μM (Fig. 7D) and 17.25 ± 0.34 to 13.04 ± 0.57 μM (Fig. 7E), respectively. In addition, the compounds alone have no effect on nitrite level of RAW 264.7 cells. From the results, all tested compounds from *C. wanenlueanga* decreased the nitrite level in LPS-induced RAW 264.7 cells.

The effects of isolates from *C. wanenlueanga* on the proinflammatory cytokine expression (TNF-α and IL-6) in LPS-stimulated RAW 264.7 cells were evaluated using ELISA. The cells triggered with LPS in every test showed elevation in TNF-α and IL-6 level to approximately 600 and 300 pg/mL, respectively, compared to the control (Fig. 7). The treatment with compound **1** at 4 μM significantly reduced TNF-α level from 634.53 ± 21.64 to 430.53 ± 34.59 pg/mL and IL-6 levels from 313.61 ± 15.48 to 155.28 ± 10.91 pg/mL (Fig. 7A), respectively. Compound **2** significantly downregulated TNF- α and IL-6 levels at concentrations more than 0.25 and 0.5 μM, respectively (Fig. 7B). Compound **3** at concentrations ≥ 2 μM significantly reduced the TNF-α level, and IL-6 level was significantly reduced after treatment with compound **3** at concentration ≥ 1 μM (Fig. 7C). Compounds **4** and **5** significantly decreased the TNF-α and IL-6 levels at

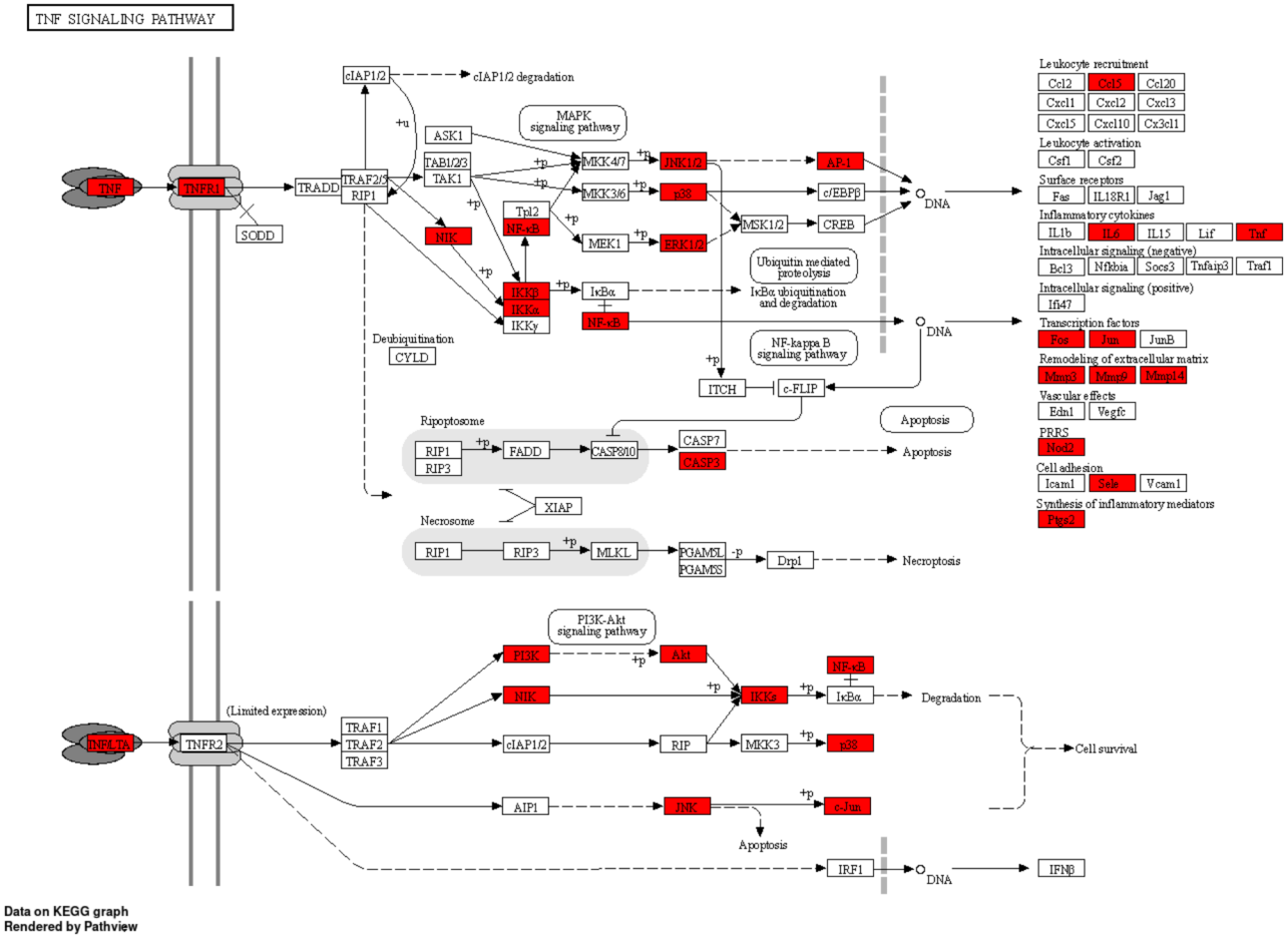

Fig. 6 The distribution of genes in the TNF signaling pathway. → represents the activation effect, T arrows represent the inhibition effect, and dashed lines represent activation or inhibition effects. The intersection genes are highlighted in red

highest concentration of 8 μM (Figs. 6E and 7D). The isolated compounds alone had no effect on the TNF-α and IL-6 levels. These findings showed that the isolated compounds inhibited the expression of TNF-α and IL-6 in a concentration-dependent manner.

Discussion

Numerous studies of plants in the *Curcuma* genus have revealed promising therapeutic potential. For instance, the well-known *C. longa* was proved to exhibit a range of biological activities such as antioxidant, anti-inflammation, hepatoprotective and anticancer activities, etc., largely attributed to curcumin and its derivatives [10, 28]. *C. latifolia* was demonstrated to ameliorate inflammatory pain in mice [12]. *C. caesia* was known for antibacterial, anti-inflammatory, anti-fungal activities, etc [29]. *C. aeruginosa* also possessed anti-cancer, antioxidant, anti-inflammatory and analgesic activities [30]. Given the ethnomedical uses of *C. wanenlueanga* in treating inflammation of joints, muscles and tendons [17], the ethanolic extract of *C. wanenlueanga* rhizomes was hypothesized to exhibit anti-inflammatory activity. Since certain inflammatory responses may rely on oxidative stress [31], a quick, easy, and affordable DPPH assay was used for screening of antioxidant activity. Additionally, the Griess assay, which measures scavenging activity toward nitric oxide, an inflammation mediator [32], was also employed. The ethanolic extract *C. wanenlueanga* rhizomes demonstrated antioxidant activities via DPPH and Griess assays. The crude extract also showed anti-inflammatory activity in LPS-stimulated RAW 264.7 macrophage cells, indicating the ability of the extract to suppress NO releases. These results show the consistency between antioxidant activities and anti-inflammatory activity in LPS-induced RAW 264.7 cells. Considering that oxidative stress and inflammation are related to joint, muscle and tendon disorders, such as rheumatoid arthritis, tendonitis, tendinopathy, etc [33, 34]., the findings suggest the correlation between the biological activities of *C. wanenlueanga* and its ethnomedical uses.

In modern medicine, studies on single drug compounds have rapidly advanced due to the development in

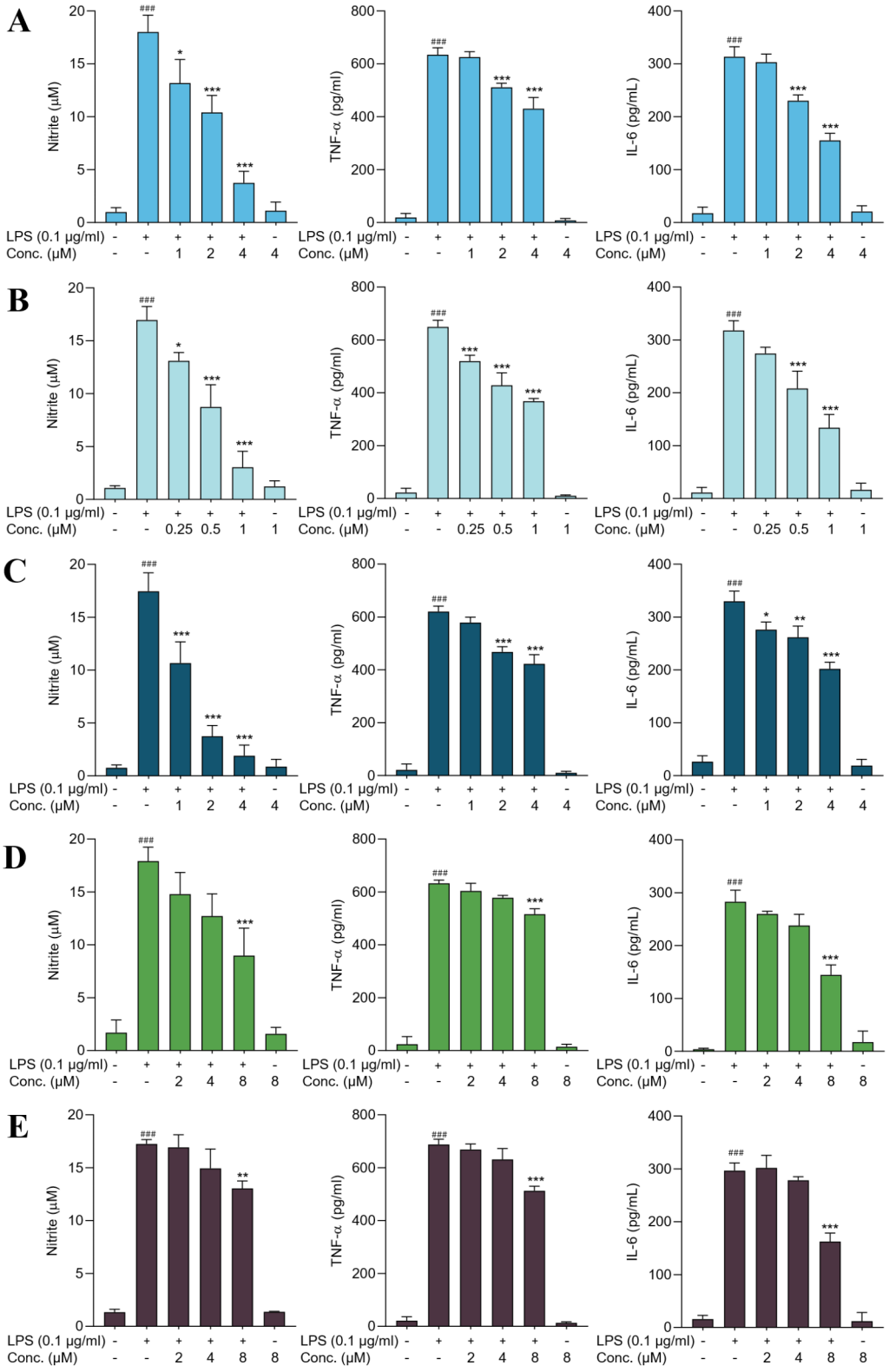

Fig. 7 (See legend on next page.)

(See figure on previous page.)

Fig. 7 The effects of the isolated compounds from *C. wanenlueanga* on the release of inflammatory mediators in activated macrophages. Griess and ELISA results of curcumin (**A**), demethoxycurcumin (**B**), dihydrodemethoxycurcumin (**C**), curcumenone (**D**), and zedoarondiol (**E**) on the releasing of NO, TNF-a and IL-6 in LPS-induced RAW 264.7 cell. Data are presented as mean value ± SD ($n = 3$). The statistical differences were analyzed using one-way ANOVA followed by Bonferroni post hoc test. ###$p < 0.001$ denotes significant difference between vehicle vs. LPS group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ denotes statistically significant between LPS vs. treatment groups

structure-activity guided chemistry, combinatorial chemistry, and in silico drug design [35], resulting in development of various potent therapeutic agents for treatment of specific diseases. However, medicinal plant extracts, which have been used since ancient times to alleviate symptoms of various ailments, continue to attract attention due to their numerous benefits. Plant extracts are more affordable because they can be derived from local herbs and require simple, low-cost extraction procedures. In contrast, single drug compounds are expensive due to extensive production steps, including synthesis and purification [36]. Plant extracts also contain a variety of compounds that can act on multiple targets, leading to synergistic interactions that enhance efficacy and help overcome drug resistance mechanisms. In contrast, a single drug compound typically affects only one target [37]. To utilize herbal extracts effectively, one should specify bioactive markers, which are the components responsible for the specific bioactivity of the plant extract. In this study, bioassay-guided isolation was conducted to identify bioactive markers, which are responsible for the anti-inflammatory activity of *C. wanenlueanga* extract. According to the results, the EtOAc partition from *C. wanenlueanga* rhizome extract showed the greatest antioxidant activities on both DPPH and Griess assays. The potential of the EtOAc partition was further confirmed as it showed greater anti-inflammatory activity than that of the crude extract. This indicates that most bioactive compounds are contained in the EtOAc partition. After separating the EtOAc partition, the antioxidant activities of fractions A_3 and A_4 were closed together and surpassed the activity of fractions A_1–A_2. Fractions A_3 and A_4 also exhibited anti-inflammatory activity on LPS-stimulated RAW 264.7 cells with comparable IC_{50} values to dexamethasone, indicating strong anti-inflammatory activity. Thus, fractions A_3 and A_4 were further separated to yield compounds **1–5**, which were expected to possess anti-inflammatory activity.

Network pharmacology was applied to predict the mechanism of action of the isolated compounds and to explore the interactions among bioactive compounds, biological systems, and diseases. Protein targets were predicted using the Swiss Target Prediction, Similarity ensemble approach (SEA) and PharmMapper databases, which compare query compounds with those of known bioactivities in their libraries [38]. These predictions are based on the concept that compounds (ligands) with structural similarities are likely to exhibit binding potential to similar protein targets. Given that RA is a disease characterized by chronic joint inflammation [34], it was used as a model to identify target genes involved with inflammatory processes. Overlapping genes between the isolated compounds and RA-related targets suggest that these compounds may play an important role in improving RA symptoms. PPI network analysis showed the relationship between the targets of the isolated compounds and inflammation, while analysis by using the cytoHubba plug-in identified key targets associated with RA. For instance, TNF-α and IL-6 are well known inflammatory cytokines involved in various inflammatory responses and are strongly associated with the development of RA [39]. MMP9, a zinc-dependent endopeptidase, contributes to RA progression by degrading the extracellular matrix (ECM), which subsequently releases cytokines associated with inflammation [40]. STAT3 activates downstream of IL-6 via the gp30 receptor, alters synovial cell growth, leading to chronic arthritis [41]. CASP3 is involved in the caspase 3/gasdermin E-mediated pathway, activated by TNF-α, which induces pyroptosis and the release of proinflammatory cytokines such as interleukin-1β [42].

GO and KEGG enrichment analyses indicated that the bioactive compounds from *C. wanenlueanga* regulate inflammatory responses and modulate reactions to bacterial molecules, lipopolysaccharides, and oxidative stress. Moreover, these compounds demonstrated significant modulation of the TNF signaling pathway, a critical contributor to the inflammatory response and joint damage characteristic of RA. In the TNF signaling pathway, TNF-α binds to TNFR1 and TNFR2, initiating the recruitment of adaptor proteins that activate transcription factors and apoptotic pathways. TNFR1 is predominantly associated with pro-inflammatory responses, while TNFR2 is implicated in the activation of fibroblast-like synoviocytes (FLS), which are critical for synovial inflammation in RA [43]. The central role of TNF-α in RA is evidenced by the clinical efficacy of TNF-α inhibitors in reducing symptoms and slowing disease progression [44]. Our KEGG pathway predictions revealed that the compounds isolated from *C. wanenlueanga* could modulate multiple genes within the TNF signaling pathway, indicating their potential to modulate inflammatory responses. Moreover, these compounds exhibit effects beyond inflammation and oxidative stress, addressing mechanisms such as bacterial infections [45]. Thus, the bioactive compounds from *C. wanenlueanga* hold

potential to alleviate RA symptoms through a holistic, multi-target therapeutic approach.

To confirm that bioactive compounds from *C. wanenlueanga* can regulate inflammatory response, Griess assay for NO and ELISAs for TNF-α and IL-6 were performed on LPS-induced RAW 264.7 cells. The results indicate that compounds **1–3** showed inhibitory effect on the productions of NO, TNF-α, and IL-6 as reported in previous studies [45–50]. Compound **4** was found to inhibit the production of NO, which aligns with a previous report [52]. However, this is the first time that compound **4** was examined to regulate TNF-α, and IL-6 productions, and compound **5** showed the inhibitory effect on NO, TNF-α, and IL-6 productions. Previous studies revealed that some of the isolated compounds can modulate inflammatory responses through specific mechanisms. Curcumin (**1**), as widely reported, modulates inflammatory responses through several pathways, including NF-κB, MAPK, and JAK/STAT pathways [53]. Demethoxycurcumin (**2**) has been reported to modulate the NLRP3 inflammasome and NF-κB pathway [54]. Zedoarondiol (**5**) has been shown to affect the NF-κB pathway by reducing the phosphorylation of key proteins such as JNK, IKK, ERK, and p38 MAPK in activated microglia [51]. In addition, the ability to regulate inflammatory response of the isolated compounds is related to their chemical structures. A recent review indicates that p-hydroxyphenyl moiety and conjugated double bonds on curcuminoids play an important role on the antioxidant and NO inhibition [55]. The α,β-unsaturated ketone part also contributes to NO inhibitory activity, and responsible for deactivation of NF-κB pathway by blocking IKKα and IKKβ activation [55, 56]. Hence, the ability of compounds **1–5** to regulate NF-κB pathway may attributed from α,β-unsaturated ketone moiety in their structures. These suggest the consistency between network pharmacology and the experimental results, highlighting its versatility as a powerful tool for predicting the effects and mechanisms of bioactive compounds on multiple targets. However, no detailed studies have yet explored the mechanisms of dihydrodemethoxycurcumin (**3**) and curcumenone (**4**), highlighting the need for further investigation.

The anti-inflammatory activity of the isolated compounds indicates that these compounds are suitable for use as bioactive markers, and their quantity could reflect the anti-inflammatory efficacy of *C. wanenlueanga* extract. In addition, the renown curcuminoids **1** and **2** appear as chemical markers for *C. longa* in the British [57], European [58], United State [59], Indian [60], Chinese [61] and Thai [62] pharmacopoeias, indicating that *C. wanenlueanga* and *C. longa* share mutual chemical markers and supporting the use of curcuminoids as anti-inflammatory markers of *C. wanenlueanga*. Many studies also revealed that sesquiterpenoids **4** and **5** are common in *Curcuma* genus as they were primarily found in many *Curcuma spp.*, such as *C. zedoaria* [63], *C. comosa* [64], *C.aromatica* [65], *C. elata* [66], *C. dedoaroides* [67], etc. Nevertheless compounds **4** and **5** are not acknowledged as much as compounds **1–3**, their anti-inflammatory activity partly contributes to the activity of *C. wanenlueanga* extract. Therefore, using curcuminoids **1–3** in combine with **4–5** could better reflect the quality of *C. wanenlueanga* extract. Further exploration on the chemical fingerprint of *C. wanenlueanga* could provide more information related to its bioactivity. In addition, more investigation on synergistic effects is recommended to provide the underlying interactions between chemical constituents of *C. wanenlueanga* extract.

Conclusion

This is the first time that *C. wanenlueanga* rhizome was demonstrated to show antioxidant and anti-inflammatory activities. Five chemical constituents including curcumin, demethoxycurcumin, dihydrodemethoxycurcumin, curcumenone, and zedoarondiol were isolated by bioassay-guided fractionation. All isolated compounds exhibit anti-inflammatory activity by down-regulating the production of NO, TNF-α, and IL-6 in LPS-induced RAW264.7 cells. This study supports the traditional uses of *C. wanenlueanga* against inflammation and suggests the use of its anti-inflammatory constituents for quality assessment of *C. wanenlueanga*, which could be a part in the development of herbal products from *C. wanenlueanga*.

Abbreviations

ALB	Albumin
ANOVA	Analysis of variance
AP-1	Activator protein 1
AR	Analytical reagent
ATCC	American Type Culture Collection
BCL2	B-cell lymphoma 2
n-BuOH	1-butanol
c/EBPβ	CCAAT-enhancer-binding proteins
CASP3	Caspase-3
^{13}C-NMR	Carbon nuclear magnetic resonance
CREB	CAMP response element-binding protein
DCM	Dichloromethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERKL	Extracellular signal-regulated kinase
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fetal bovine serum
GO	Gene ontology
HIF1A	Hypoxia-inducible factor 1-alpha
^{1}H-NMR	Proton nuclear magnetic resonance
HPTLC	High performance thin-layer chromatography
HRTOFMS	High-resolution time-of-flight mass spectrometry
IKK	Inhibitor of κB kinase
IL-1	Interleukin-1

IL-2	Interleukin-2
IL-6	Interleukin-6
IFN-γ	Interferon-gamma
JUN	Transcription factor Jun
JNK	c-Jun N-terminal kinase
KEGG	Kyoto encyclopedia of genes and genomes
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
MMP9	Matrix metalloproteinase-9
MNTC	Maximum non-toxic concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIK	NF-κB-inducing kinase
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
PBS	Phosphate buffer saline
PPI	Protein–protein interactions
RA	Rheumatoid arthritis
RIP1	Receptor-interacting protein kinase 1
SD	Standard deviation
SiO_2	Silica gel
TNF-α	Tumor necrosis factor-alpha
SNP	Sodium nitroprusside
STAT3	Signal transducer and activator of transcription 3
TAK1	Transforming growth factor-β-activated kinase 1
TLC	Thin-layer chromatography
TNFR1	Tumor necrosis factor receptor 1
TRADD	TNFR1-associated death domain
TRAF	TNF receptor-associated factors
VLC	Vacuum liquid chromatography

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12906-025-04884-0.

Supplementary Material 1

Acknowledgements

We would like to thank Pharmaceutical Research Instrument Center (PRICE), Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand, for providing support with scientific instruments. This research project is supported by the Second Century Fund (C2F), Chulalongkorn University. We also acknowledge Associate Professor Thatree Phadungcharoen for identification of the plant species.

Author contributions

WT and HH performed the methodology, investigation, data curation, formal analysis, visualization, and they drafted the original manuscript. SSJ and PWDW provided resources for computational analysis. SS, PT, PWDW, and BS supervised and conducted critical reviews. WT, HH, PWDW and SS edited the manuscript. SS is the principal investigator who conceptualized the project. All authors read and approved the final manuscript.

Funding

This research has received funding support from the National Science, Research and Innovation Fund (NSRF) via the Program Management Unit for Human Resources & Institutional Development, Research and Innovation [grant number B13F660137].

Data availability

The data used to support the findings of this study are included within the article.

Declarations

Ethics approval and consent to participate

C. wanenlueanga rhizomes were legally collected from private land in Uttaradit Province, Thailand, with the landholder's permission. The plant sample was identified by Associate Professor Thatree Phadungcharoen, the taxonomist at Faculty of Pharmaceutical Sciences, Chulalongkorn University. A voucher specimen (SS-894) was deposited at the Museum of Natural Medicines, Chulalongkorn University, Bangkok, Thailand. All experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

SS and PT are the co-founders of Herb Guardian Co., Ltd. WT reports a relationship with Herb Guardian Co., Ltd. for funding. The remaining authors declare no competing interests in relation to this study.

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Received: 6 November 2024 / Accepted: 4 April 2025
Published online: 17 April 2025

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