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Research article

Phytochemical characterization of major constituents in Thai traditional formulation for knee poultice (Ya-Pok-Dud-Pid) and their inhibition of nitric oxide production activity

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Abstract

Importance of the work: The Thai traditional formulation for a knee poultice (Ya-Pok-Dud-Pid) has been used for the treatment of knee inflammation. Basic information is required for quality assessment of this traditional approach, regarding its major active components with anti-inflammatory activities.

Objectives: To isolate and identify the major components of a Ya-Pok-Dud-Pid formulation from the plant ingredients and to investigate the inhibitory effects on the lipopolysaccharide-induced nitric oxide release of its ingredients and isolated compounds.

Materials and Methods: The tincture of the Ya-Pok-Dud-Pid formulation and methanolic extracts of each plant sample were subjected to analysis using ultra-high performance liquid chromatography coupled with diode array detection, as well as using thin-layer chromatography. The major components were isolated and their chemical structures were elucidated. Inhibitory effects on lipopolysaccharide-induced nitric oxide release were investigated using the murine macrophage RAW 264.7 cell line.

Results: The phytochemical investigation resulted in the identification of (*E*)-4-(3',4'-dimethoxyphenyl) but-3-en-1-ol (compound D, **1**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**), (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**), piperine (**4**), zerumbone (**5**), zerumbone epoxide (**6**), 6',7'-dihydroxybergamottin (**7**), oxypeucedanin hydrate (**8**), curcumin (**9**), β-asarone (**10**), colchicine (**11**), plumbagin (**12**) and 6-gingerol (**13**). The Ya-Pok-Dud-Pid extract was anti-inflammatory, with a half maximal inhibitory concentration (IC_{50}) of 59.86 µg/mL. Based on the crude 40% ethanolic extracts, *Zingiber zerumbet* had the highest activity with an IC_{50} of 15.45 µg/mL, followed by *Crinum asiaticum*, *Curcuma aromatica*, *Zingiber officinale, Zingiber cassumunar and Globba variabilis*. Furthermore, zerumbone (**5**) had the highest activity with an IC_{50} of 0.88 µg/mL followed by plumbagin (**12**), curcumin (**9**), zerumbone epoxide (**6**), colchicine (**11**), DMPBD (**3**), piperine (**4**), D-acetate (**2**) and compound D (**1**), while the NG-methyl-L-arginine acetate salt (positive control) had an IC_{50} of 17.16 µg/mL.

Main findings: The active components were identified in the Ya-Pok-Dud-Pid formulation, which could provide chemical markers for quality assessment and contribute valuable information for further pharmaceutical development.

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Introduction

Osteoarthritis is the most common form of arthritis, characterized by chronic pain and loss of mobility, with approximately 7.6% (595 million people) of the global population reported to have osteoarthritis in 2020, representing an increase of 132.2% in total cases since 1990 (Steinmetz et al., 2023). Knee osteoarthritis is the most prevalent type and was estimated to affect 642 million individuals in 2050 (Steinmetz *et al.,* 2023). The economic burden on patients and society is high and there are no current licensed drugs with proven disease-modifying activity for osteoarthritis; thus, management of osteoarthritis is focused on improving pain, disability and quality of life (Hawker, 2019).

Pathological mechanisms in osteoarthritis are impacted by synovial, bone and cartilage, with cartilage being the main target, where cytokine cascades and the production of inflammatory mediators in the cartilage drive the imbalance of metabolic and degradative signals, activating progressive degeneration (Krasnokutsky et al., 2008). Osteoarthritis patients have increased levels of inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-a (TNF-a), which in turn decrease anabolic collagen synthesis and decrease catabolic (including matrix metalloproteinases or MMP) and other inflammatory mediators such as IL-8, IL-6, prostaglandin E2 and nitric oxide (Krasnokutsky et al., 2008).

Herbal drugs in traditional medicine have a long history of being used for treatment of knee inflammation. For example, a Thai traditional knee poultice patch called Ya-Pok-Dud-Pid has been used for treatment of knee osteoarthritis (Department of Thai Traditional and Alternative Medicine Development, 2016). It is a 40% ethanolic tincture of a polyherbal mixture of *Zingiber cassumunar* Roxb. rhizomes (30%), camphor (15%), *Citrus hystrix* DC. fruit peel (3%), *Piper nigrum* L. fruit (3%), *Curcuma aromatica* Salisb. As well as tubers (3%), *Globba variabilis* subsp. *variabilis* rhizomes (3%), *Alpinia galanga* (L.) Willd rhizomes (3%), *Plumbago indica* L. roots (3%), *Piper retrofractum* Vahl fruit (3%), *Putranjiva roxburghii* Wall. leaves (3%), *Tamarindus indica* L. leaves (3%), *Senegalia pennata* (L.) Maslin leaves (3%), *Zingiber zerumbet* (L.) Roscoe ex Sm. rhizomes (3%), *Zingiber officinale* Roscoe rhizomes (3%), *Cleome viscosa* L. leaves (3%), *Acorus calamus* L. roots (3%), *Gloriosa superba* L. roots (3%), *Crinum asiaticum* L. leaves (3%), *Tradescantia zebrina* Bosse leaves (3%) and salt (3%), according to Department of Thai Traditional and Alternative Medicine Development (2016).

Other studies have reported that the Ya-Pok-Dud-Pid remedy and its herbal ingredients have scientifically proven anti-inflammatory activities and osteoarthritis treatment. For example, it improved knee osteoarthritis symptoms in parameters of visual analog scales, time up and go test, active knee flexion, passive knee flexion, chair sit and reach and the Western Ontario and McMaster Universities Arthritis Index, comparable to a NSAID diclofenac gel (Saereewat et al., 2024). *Z. cassumunar* provides anti-inflammatory activity via cyclooxygenase-2 (COX-2) (Jeenapongsa et al., 2003; Koontongkaew et al., 2013), lipoxygenase (Jeenapongsa et al., 2003) and MMP (Koontongkaew et al., 2013) pathways and relieves knee osteoarthritis (Niempoog et al., 2012; Joshi and Phansopkar, 2022). Camphor was effective in reducing the pain from osteoarthritis of the knee (Cohen et al., 2003). Ethanolic extracts of *Z. zerumbet, Z. cassumunar, G. variabilis* (syn. *G. malaccensis*), *P. nigrum, Z. officinale*, *P. indica, P. longum, C. asiaticum, P. roxburghii, C. aromática, A. galanga* and *C. viscosa* could inhibit nitric oxide production in RAW 264.7 cells (Anuthakoengkun and Itharat, 2014).

Despite the proven pharmacology of this drug, quality consistency is a crucial concern, since the quality of herbal medicines may be affected by many factors such as seasonality, harvesting time, cultivation area, post-harvesting processing, adulterants or substitutes of raw materials and procedures in extraction and preparation (Li et al., 2008). Quality assessment of herbal drugs could be carried out using appropriate chemical markers combined with chemical fingerprinting.

Therefore, the current study carried out the phytochemical investigation of a Ya-Pok-Dud-Pid formulation, involving the isolation of the major components of the main ingredients and the elucidation of their chemical structures, as well investigating their inhibitory effects on lipopolysaccharide-induced nitric oxide release using murine macrophage cell line. The results should contribute valuable information to the quality assessment of this drug and further pharmaceutical developments.

Materials and Methods

Plant materials and chemicals

The Thai traditional formulation called Ya-Pok-Dud-Pid (batch no. 220001, 25 Aug 2022) and its herbal ingredients were obtained from the Herbal Medicines and Products Manufacturing Unit, manufactured under a good manufacturing

practice (GMP) by U-thong Hospital, Suphanburi province, Thailand. The herbal ingedients were: *Zingiber cassumunar* rhizomes, camphor, *Citrus hystrix* fruit peel, *Piper nigrum* fruit, *Curcuma aromatica* tubers, *Globba variabilis* rhizomes, *Alpinia galanga* rhizomes, *Plumbago indica* roots, *Piper retrofractum* fruits, *Putranjiva roxburghii* leaves, *Tamarindus indica* leaves, *Senegalia pennata* leaves, *Zingiber zerumbet* rhizomes, *Zingiber officinale* rhizomes, *Cleome viscosa* leaves, *Acorus calamus* roots, *Gloriosa superba* roots, *Crinum asiaticum* leaves, *Tradescantia zebrina* leaves and salt,. All plants were taxonomically identified based on their morphological characters. Dried plant materials (*Z. cassumunar* rhizomes, *Z. officinale* rhizomes, *P. retrogractum* fruits, *P. nigrum* fruits, *C. hystrix* fruit peels and *A. calamus* rhizomes) were authenticated using microscopic and macroscopic characters as described in the Thai Herbal Pharmacopoeia (Department of Medical Sciences, Ministry of Public Health, Thailand, 2021). The dried samples were kept in air-tight plastic bags at room temperature.

NG-methyl-L-arginine acetate salt (L-NMMA, purity ≥ 98%, based on thin-layer chromatography, TLC), (±)-camphor (purity 96%) and colchicine (purity > 95%, based on high-performance liquid chromatography, HPLC) were purchased from Sigma-Aldrich (USA). Plumbagin (purity > 98%) and curcumin (purity > 97%) were purchased from TCI (Japan). 6-Gingerol (purity 98%), 6-shagoal (purity 98%) and alpinetin (purity 98%) were purchased from ChemFaces (China). HPLC grade methanol and formic acid were purchased from Fisher Scientific England). Deionized water was purified using an Ultra Clear system (Siemen Water Technologies Corp., USA). All solvents and reagents were of analytical grade, if not stated otherwise.

The Griess reagent was prepared by dissolving 2.3 mL of orthophosphoric acid (Fisher, England), 1 g of sulfanilamide (Sigma-Aldrich, USA), 0.1 g of N-(1-naphtyl) ethylenediamine dihydrochloride (Sigma-Aldrich, USA) in deionized water and adjusted to 100 mL. The reagent was kept in -20°C and protected from light.

Phytochemical characterization and isolation of major components

Thin-layer chromatography

Each plant sample was extracted using methanol at a concentration of 10 mg/mL with sonication for 30 min at room temperature. The 40% ethanolic tincture of the Thai traditional formulation called Ya-Pok-Dud-Pid and the crude methanolic herbal extracts were subjected to TLC performed on silica gel 60 F_{254} (0.2 mm; Merck; Germany) with *n*-hexane-ethyl acetate (7:3, v/v) as the mobile phase, with spraying using anisaldehyde-sulfuric reagent.

Ultra-high performance liquid chromatography

Analysis using ultra-high performance liquid chromatography coupled with diode array detection (UHPLC-DAD) was performed on a Thermo Fisher Scientific Inc. Vanquish UHPLC system (equipped with a binary pump F, a split sampler FT, column compartment H and diode array detector FG). Separation was carried out using a Hypersil GOLD C18 column (100 × 2.1 mm i.d., 1.9 µm; ThermoScientific; USA). The mobile phases were: (A) 0.1% formic acid in water; and (B) methanol. A mobile phase time program was set up with a linear increase from 15% B to 60% B in A for 35 min, 60% B in A for 15 min, 60% B to 75% B in A for 15 min and 100% B for 5 min Before each injection, the column was equilibrated with 15% B in A for 10 min. The column temperature was controlled at 25°C with a constant flow rate of 0.5 mL/min. DAD was set at 254 nm and the injection volume setting was 2 µL for all samples.

*Isolation of (E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), (E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**) and (E)-1-(3',4'-dimethoxyphenyl) butadiene (DMPBD, **3**) from Z. cassumunar*

A sample (200 g) of dried powdered *Z. cassumunar* rhizomes was macerated with *n*-hexane three times with occasional shaking for 72 hr at room temperature. The solution was filtered, combined and evaporated dryness using rotary evaporation, yielding 2.44 g of crude extract. The extract was subjected to column chromatography (silica gel, 0.063–0.200 mm) using 5–30% ethyl acetate in *n*-hexane. Fractions were monitored using TLC with *n*-hexane: ethyl acetate (7:3, volume per volume, v/v) as the mobile phase and combined. The column chromatography was repeated using the same solvent mixture until pure compounds were achieved, yielding 750 mg of (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**), 57 mg of (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**) and 87 mg of (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**) (Seaho et al., 2024; Seaho et al., 2025).

*Isolation of piperine (**4**) from P. nigrum*

A sample (15 g) of dried powdered *P. nigrum* was g extracted using ethanol 150 mL and a Soxhlet apparatus.

The solution was filtered and evaporated using a rotary evaporator. Then, 10% NaOH in ethanol 10 mL was added and filtered. Piperine (**4**) was crystallized from the solution and washed with cold ethanol. Recrystallization using ethanol was carried out to produce pure piperine (**4**) 0.25 g.

*Isolation of zerumbone (**5**) and zerumbone epoxide (**6**) from Z. zerumbet*

A sample (200 g) of dried powdered *Z. zerumbet* rhizomes was macerated with *n*-hexane three times with occasional shaking for 72 h at room temperature. The solution was filtered, combined and evaporated to dryness using rotary evaporation, yielding crude 2.4 g of extract. The extract was subjected to column chromatography (silica gel, 0.063-0.200 mm) using 10% ethyl acetate in *n*-hexane. Fractions were monitored using TLC with *n*-hexane:ethyl acetate (9:1, v/v) as the mobile phase and combined. Final cleaning up was achieved using Sephadex LH-20 eluted with methanol, yielding 139 mg of zerumbone (**5**) and 45 mg of zerumbone epoxide (**6**).

*Isolation of 6',7'-dihydroxybergamottin (**7**) and oxypeucedanin hydrate (**8**) from C. hystrix*

A sample (200 g) of dried powdered *C. hystrix* fruit peel was macerated with ethyl acetate three times with occasional shaking for 72 h at room temperature. The solution was filtered, combined and evaporated to dryness using rotary evaporation, yielding crude 12.28 g of extract. The extract was subjected to column chromatography (silica gel, 0.063-0.200 mm) using 50% ethyl acetate in *n*-hexane. Fractions were monitored using TLC with *n*-hexane:ethyl acetate (5:5, v/v) as the mobile phase and combined. The column chromatography was repeated with the same solvent mixture until the pure compound was achieved, yielding 90 mg of 6',7'-dihydroxybergamottin (**7**). Another fraction was subjected to column chromatography using an *n*-hexane-to-dichloromethane-to-methanol ratio of 7:3:0.5 (volume per volume per volume) as the mobile phase. The column chromatography was repeated with the same solvent mixture until the pure compound was achieved, yielding 40 mg of oxypeucedanin hydrate (**8**).

*Isolation of curcumin (**9**) from C. longa*

A sample (327 g) of dried powdered *C. longa* was macerated with 1 L of ethanol three times with occasional shaking for 72 h at room temperature. The solution was filtered, combined and evaporated to dryness using rotary evaporation, yielding crude 38 g of extract. The extract was subjected to column chromatography (silica gel, 0.063–0.200 mm) using 50% dichloromethane in *n*-hexane. Fractions were monitored using TLC with an *n*-hexane-to-dichloromethane ratio of 4:6 (volume per volume) as the mobile phase and combined. The column chromatography was repeated with the same solvent mixture. Final purification was achieved using Sephadex LH-20 eluted with methanol. The processes were repeated until the pure compound was achieved, yielding 61.8 mg of curcumin (**9**).

Structure elucidation

Nuclear magnetic resonance spectroscopy and mass spectrometry

All compounds were assessed for purity based on TLC, HPLC and the melting point. The nuclear magnetic resonance (NMR) spectra were recorded on either a Bruker Avance NEO 400 NanoBay (Germany) or a Bruker Avance III HD 600 MHz spectrometer (Germany), using the standard Bruker pulse programs. The samples were dissolved in 0.6 mL of chloroform-d_1 (99.8% D; Merck KGaA; Germany) or methanol-d_4 (99.8% D; Euriso-Top; France) or dimethylsulfoxide-d_6 (99.8% D; Euriso-Top; France). The infrared (IR) spectra were recorded on a Bruker Alpha II (Diamond ATR) compact FTIR spectrometer (Germany). Mass spectra were recorded on a high-resolution Orbitrap mass spectrometer (Thermo Scientific Orbitrap Exploris™ 120; USA) based on direct infusion heated-electrospray ionization in positive ionization mode, as well as in negative mode. High resolution mass measurements were performed within the selected mass range of *m/z* 100–2000. The spray voltage was set in static mode with a positive ion setting of 3500 V and negative ion setting of 2500 V. The nitrogen gas mode was static with flow settings of sheath gas 25 Arb, auxiliary gas 5 Arb and sweep gas 0 Arb. The ion transfer tube temperature was 320°C and the vaporizer temperature was 75°C.

*(E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**) (Seaho et al., 2024; Seaho et al., 2025)*

Proton nuclear magnetic resonance (^{1}H NMR): (600 MHz, chloroform-d_1), *d* (ppm) = 6.95 (d, *J* = 1.7 Hz, 1H, Ar-*H*), 6.91 (dd, *J* = 8.2, 1.6 Hz, 1H, Ar-*H*), 6.83 (d, *J* = 8.2 Hz, 1H, Ar-*H*), 6.46 (d, *J* = 15.8 Hz, 1H, C*H* = CHCH$_2$), 6.12–6.07 (m, 1H, CH=C*H*CH$_2$), 3.92 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 3.78 (t, *J* = 6.3 Hz, 2H, CH_2OH), 2.50 (q, *J* = 6.6 Hz, 2H, CHCH_2CH$_2$).

Carbon-13 nuclear magnetic resonance (^{13}C NMR): (150 MHz, chloroform-d_1), *d* (ppm) = 149.0, 148.5, 132.4, 130.4, 124.4, 119.1, 111.1, 108.6, 62.1, 55.9, 55.8, 36.4.

Fourier-transform infrared spectroscopy (attenuated total reflectance) (FT-IR (ATR): *n* (cm^{-1}) = 3385 (br), 2934, 2836, 1602, 1511, 1259, 1231, 1022.

Quadrupole-Orbitrap high resolution mass spectrometry (Q-Orbitrap HRMS): *m/z* $[M+Na]^+$, calcd for $C_{12}H_{16}O_3Na$: 231.0997, found: 231.0993.

*(E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**) (Seaho et al., 2024; Seaho et al., 2025)*

^{1}H NMR (600 MHz, chloroform-d_1), *d* (ppm) = 6.93 (d, *J* = 1.9 Hz, 1H, Ar-*H*), 6.90 (dd, *J* = 8.2, 1.9 Hz, 1H, Ar-*H*), 6.83 (d, *J* = 8.2 Hz, 1H, Ar-*H*), 6.43 (d, *J* = 15.8 Hz, 1H, C*H*=CHCH$_2$), 6.08 – 6.04 (m, 1H, CH=C*H*CH$_2$),4.20 (t, 2H, CH_2OAc), 3.92 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 2.55 (qd, *J* = 6.9, 1.3 Hz, 2H, CH = CHCH_2), 2.08 (s, 3H, OCOCH_3).

^{13}C NMR (DEPTQ, 150 MHz, chloroform-d_1), *d* (ppm) = 171.2, 149.0, 148.6, 132.1, 130.4, 123.6, 119.1, 111.2, 108.6, 63.9, 55.9, 55.8, 32.3, 21.0.

FT-IR (ATR): *n* (cm^{-1}) = 2955, 2935, 1734, 1512, 1462, 1230, 1024.

Q-Orbitrap HRMS: *m/z* $[M+Na]^+$, calcd for $C_{14}H_{18}O_4Na$: 273.1103, found: 273.1097.

*(E)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**) (Seaho et al., 2024; Seaho et al., 2025)*

^{1}H NMR (600 MHz, chloroform-d_1), *d* (ppm) = 6.99 (d, *J* = 1.9 Hz, 1H, Ar-*H*), 6.97 (dd, *J* = 8.2, 2.0 Hz, 1H, Ar-*H*) , 6.84 (d, *J* = 8.2 Hz, 1H, Ar-*H*), 6.69 (dd, *J* = 15.5, 10.6 Hz, 1H, Ar-*H*), 6.55–6.50 (m, 2H, C*H* = C*H*), 5.34-5.31 (m, 1H, ArCH=CH_2), 5.16-5.14 (m, 1H, ArCH = CH_2), 3.94 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3).

^{13}C NMR (150 MHz, chloroform-d_1), *d* (ppm) = 149.1, 148.9, 137.3, 132.6, 130.3, 127.9, 119.9, 116.7, 111.2, 108.7, 55.9, 55.8.

FT-IR (ATR): *n* (cm^{-1}) = 3089, 2998, 2958, 1629, 1594, 1509, 1440, 1234, 1134, 1013.

Q-Orbitrap HRMS: *m/z* $[M+H]^+$, calcd for $C_{12}H_{15}O_2$: 191.1072, found: 191.1069; $[M+Na]^+$, calcd for $C_{12}H_{14}O_2Na$: 213.0891, found: 213.0888.

*Piperine (**4**) (Khan et al., 2017)*

^{1}H NMR (400 MHz, chloroform-d_1), *δ* (ppm) = 7.41 (ddd, *J* = 14.6, 8.4, 1.7 Hz, 1H, C*H*CHCO), 6.99 (s, 1H, Ar-*H*), 6.90 (d, *J* = 8.1 Hz, 1H, Ar-*H*), 6.82 – 6.68 (m, 3H, ArC*H* = C*H*, Ar-*H*), 6.45 (d, *J* = 14.7 Hz, 1H, C = C*H*CO), 5.98 (s, 2H, -OCH_2O-), 3.61-3.59 (m, 4H, -N(CH_2)(CH_2), 1.71-1.57 (m, 6H, -CH_2CH_2CH_2-).

^{13}C NMR (100 MHz, chloroform-d_1), *δ* (ppm) = 165.4 (*C*O), 148.2 (Ar*C*), 148.1 (Ar*C*), 142.4 (*C*H = CHCO), 138.2 (Ar*C*H = C), 131.0 (Ar*C*), 125.4 (ArCH = *C*H), 122.4 (Ar*C*), 120.1 (*C*HCO), 108.4 (Ar*C*), 105.7 (Ar*C*), 101.2 (-O*C*H$_2$O-), 46.6 (CH$_2$N*C*H$_2$), 43.5 (*C*H$_2$NCH$_2$), 26.1 (*C*H$_2$), 24.6 (*C*H$_2$CH$_2$).

*Zerumbone (**5**) (Kitayama et al. 1999; Kongkiatpaiboon et al., 2023)*

^{1}H NMR (400 MHz, chloroform-d_1), *d* (ppm) = 6.01-5.98 (m, 1H, *H*3), 5.96 (d, *J* = 16.4 Hz, 1H, *H*11), 5.84 (d, *J* = 16.4 Hz, 1H, *H*10), 5.25-5.21 (m, 1H, *H*7), 2.48-2.16 and 1.89-1.86 (m, 1H, 2H, 2H, 1H, *H*4, *H*5, *H*8), 1.78 (s, CH$_3$, *H*12), 1.52 (s, CH$_3$, *H*13), 1.18 (s, 3H, *H*14), 1.05 (s, CH$_3$, *H*15).

^{13}C NMR (100 MHz, chloroform-d_1), *d* (ppm) = 204.3, 160. 7, 148.8, 137.9, 136.2, 127.1, 124.9, 42.4, 39.4, 37.8, 29.4, 24.4, 24.1, 15.2, 11.7.

FT-IR (ATR): *n* (cm^{-1}) = 3025, 2963, 2919, 2857, 1652, 1638, 1455, 1429, 1385, 1363, 1262, 1183, 1103, 964, 907, 827, 697, 628.

Q-Orbitrap HRMS: *m/z* $[M+H]^+$, calcd for $C_{15}H_{23}O$: 219.1749, found: 219.1741; $[M+Na]^+$, calcd for $C_{15}H_{22}ONa$: 241.1568, found: 241.1558.

*Zerumbone epoxide (**6**) (Kitayama et al. 2001; Kongkiatpaiboon et al., 2023)*

^{1}H NMR (400 MHz, chloroform-d_1), *d* (ppm) = 6.12-6.04 (m, 3 x 1H, *H*3, *H*10 and *H*11 of vinyl proton), 2.72 (d, *J* = 11.1 Hz, 1H, *H*8), 2.47-2.36 (m, 2H, *H*5), 2.28-2.23 (m, 1H, *H*4), 1.90 (d, *J* = 14.0 Hz, 1H, *H*8¢), 1.82 (s, CH$_3$, *H*12), 1.42 (dd, *J* = 13.9 Hz, 11.3 Hz, 1H, *H*7), 1.35-1.28 (m, 1H, *H*4¢), 1.26 (s, CH$_3$, *H*13), 1.19 (s, CH$_3$, *H*14), 1.05 (s, CH$_3$, *H*15).

^{13}C NMR (100 MHz, chloroform-d_1), *d* (ppm) = 202.9, 159.5, 147.7, 139.4, 128.2, 62.8, 61.4, 42.6, 38.2, 35.9, 29.7, 24.6, 24.0, 15.6, 12.1.

FT-IR (ATR): *n* (cm^{-1}) = 3028, 3002, 2961, 2930, 2869, 1654, 1639, 1455, 1385, 1365, 1301, 1261, 1200, 1166, 1118, 1060, 970, 882, 840, 822, 764, 702, 628.

Q-Orbitrap HRMS: *m/z* $[M+Na]^+$, calcd for $C_{15}H_{22}O_2Na$: 257.1517, found: 257.1512.

*6',7'-dihydroxybergamottin (**7**) (Girennavar et al., 2006)*

^{1}H NMR (400 MHz, chloroform-d_1), *d* (ppm) = 8.17 (d, *J* = 9.8 Hz, 1H, *CH* = CHCO of pyrone), 7.61 (d, *J* = 2.4 Hz, 1H, CH = C*H*-O of furan), 7.17 (br. s, 1H, Ar-*H*), 6.97 (dd, *J* = 2.3, 0.9 Hz, 1H, C*H* = CH-O of furan), 6.29 (d, *J* = 9.8 Hz, 1H, *C*H = C*H*CO of pyrone), 5.61 (td, *J* = 6.8, 1.1 Hz, 1H, OCH$_2$*CH* = C(Me)CH$_2$), 4.97 (d, *J* = 6.8 Hz, 2H, OCH_2CH = C),

3.34 (dd, J = 10.5, 1.9 Hz, 1H, CH_2CH-OH), 2.42–2.35 (m, 1H, CH = CC*H*H¢CH_2), 2.23–2.12 (m, 1H, CH = CCH*H*¢CH_2), 2.01 (br. s, 2H, 2 x O*H*), 1.72 (br. s, 3H, CH_3), 1.68–1.57 (m, 1H, CH_2CHH¢CH-O), 1.52–1.40 (m, 1H, CH_2CH*H*¢CH-O), 1.22 (s, 3H, CH_3), 1.19 (s, 3H, CH_3).

^{13}C NMR (DEPTQ, 100 MHz, chloroform-d_1), d (ppm) = 161.3, 158.1, 152.6, 148.8, 144.9, 142.9, 139.6, 119.3, 114.2, 112.6, 107.5, 105.0, 94.3, 77.8, 73.0, 69.6, 36.5, 29.4, 26.5, 23.3, 16.6.

FT-IR (ATR): n (cm^{-1}) = 3535, 3481, 3317, 3155, 3124, 2973, 2901, 1719, 1625, 1599, 1579, 1454, 1332, 1286, 1255, 1201, 1154, 1127, 1068, 979, 929, 896, 818, 799, 749.

Q-Orbitrap HRMS: m/z $[M+H]^+$, calcd for $C_{12}H_{25}O_6$: 373.1646, found: 373.1631.

*Oxypeucedanin hydrate (**8**) (Baek et al., 2000)*

^{1}H NMR (400 MHz, methanol-d_4), d (ppm) = 8.34 (d, J = 9.8 Hz, 1H, *CH* = CHCO of pyrone), 7.74 (d, J = 2.4 Hz, 1H, CH = C*H*-O of furan), 7.16 (dd, J = 2.4, 0.9 Hz, 1H, C*H* = CH-O of furan), 7.08 (br. s, 1H, Ar-*H*), 6.22 (d, J = 9.8 Hz, 1H, *C*H = C*H*CO of pyrone), 4.76 (dd, J = 9.8, 2.4 Hz, 1H, OC*H*H¢-CHOH), 4.36 (dd, J = 9.8, 8.5 Hz, 1H, OCH*H*¢-CHOH), 3.81 (dd, J = 8.4, 2.4 Hz, 1H, OCH_2-C*H*-OH), 1.30 (s, 3H, CH_3), 1.25 (s, 3H, CH_3).

^{13}C NMR (100 MHz, methanol-d_4), d (ppm) = 163.2, 159.7, 153.7, 150.6, 146.6, 141.6, 115.1, 112.8, 108.1, 106.2, 94.5, 78.0, 75.6, 72.6, 27.2, 24.8.

FT-IR (ATR): n (cm^{-1}) = 3455, 3310, 3234, 3119, 3059, 2979, 1689, 1619, 1603, 1577, 1452,1350, 1212, 1158, 1134, 1084, 1055, 1002, 993, 902, 811, 746, 717.

Q-Orbitrap HRMS: m/z $[M+H]^+$, calcd for $C_{16}H_{17}O_6$: 305.1020, found: 305.1006.

*Curcumin (**9**) (Praveen et al., 2021)*

^{1}H NMR (400 MHz, DMSO-d_6), d (ppm) = 9.72 (br. s, 1H, O*H*), 7.55 (d, J = 15.8 Hz, 2 x 1H, ArC*H* = CHCO), 7.30 (d, J = 1.6 Hz, 2 x 1H, Ar-*H*), 7.15 (dd, J = 8.2, 1.6 Hz, 2 × 1H, Ar-*H*), 6.83 (d, J = 8.2 Hz, 2H, Ar-*H*), 6.75 (d, J = 15.8 Hz, 2H, ArCH = C*H*CO), 6.07 (s, 1H, HOC = C*H*CO), 3.83 (s, 2 × 3H, OCH_3).

^{13}C NMR (100 MHz, DMSO-d_6), d (ppm) = 183.6, 149.7, 148.4, 141.1, 126.8, 123.5, 121.5, 116.1, 111.6, 101.4, 56.1.

Inhibitory of nitric oxide production in RAW 264.7 cell line

Cell line

The macrophage cell line (RAW 264.7) was established from a tumor in a male mouse (ATCC TIB-71) and cultured in a minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. The cells were maintained at 37°C in a 5% CO_2 incubator and sub-passaged every 3 d.

Sample preparation

The 40% ethanolic Ya-Pok-Dud-Pid extract, its plant ingredients and pure compounds were dissolved in sterile dimethyl sulfoxide (DMSO) and adjusted to a 50 mg/mL concentration. L-NMMA was dissolved in DMSO, adjusted to a 50 mg/mL concentration and used as a positive control. The samples were diluted with DMEM to the desired concentration. The final concentration of DMSO was less than 1%. Each stock solution was stored at -20°C until use.

Cell viability evaluation using 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The cytotoxicity of Ya-Pok-Dud-Pid extract, its plant ingredients and pure compounds on RAW264.7 cell were determined using the 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All samples were prepared at various concentrations and diluted with DMEM to achieve final concentrations of 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL or suitable concentrations for the experiments. Initially, a confluent monolayer of RAW264.7 cells was seeded in a 96-well plate at 5 × 10^5 cell/mL and incubated in a 5% CO_2 incubator for 24 hr at 37°C. Next, different concentrations of the samples were added to each well (100 µL/well). The experiments were performed in triplicate, followed by incubation in a 5% CO_2 incubator for 24 hr at 37°C. After incubation, the supernatant (100 µL/well) was removed and 10 µL of MTT solution (5 mg/mL) was added to each well and incubated in a 5% CO_2 incubator for 3 hr at 37°C. Then, the supernatant was discarded and the formazan crystals were dissolved in 100 µL of DMSO per well. The absorbance was measured at 570 nm (OD_{570}) using a microplate reader (ThermoScientific Varioskan Flash, USA). The data were presented as mean ± SE values of the mean (SEM) and the %Cell viability was calculated using Equation 1:

$$\%\ \text{Cell viability} = (OD_{570}\ \text{of sample} / OD_{570}\ \text{of cell control}) \times 100 \quad (1)$$

Measurement of nitric oxide production

The inhibitory effect of the Ya-Pok-Dud-Pid extract, its plant ingredients and pure compounds on nitric oxide production were evaluated in RAW264.7 cells stimulated with lipopolysaccharide (LPS). The inhibition of nitric oxide production was measured using Griess reagent. First, a confluent monolayer of RAW264.7 cells was seeded in a 96-well plate at 5 × 10^5 cell/mL and incubated in a 5% CO_2 incubator for 24 h at 37°C. After incubation, the supernatant was removed and 100 µL of LPS at a concentration of 200 µg/mL was added to each well. Subsequently, varying concentrations of the test samples were added (100 µL/well). The experiments were performed in triplicate and incubated in a 5% CO_2 incubator for another 24 h at 37°C. After this incubation period, 100 µL of the supernatant from each well was transferred to a new 96-well plate, followed by the addition of 100 µL of Griess reagent to each well, with mixing for 15 min. The absorbance values were measured at 540 nm using the microplate reader. The results were reported as mean ± SEM. The %Inhibition of nitric oxide was calculated using the Equation 2.

$$\%\ \text{Inhibition of nitric oxide} = \frac{OD_{540}\ \text{of cell control} - OD_{540}\ \text{of sample}}{OD_{540}\ \text{of cell control}} \times 100 \quad (2)$$

The half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 5.01 software (GraphPad; USA).

Results

The phytochemical composition of a Thai traditional formulation for a knee poultice called Ya-Pok-Dud-Pid was investigated. Comparative analysis of the both the TLC and UHPLC results of the final formulation and of each plant ingredient identified no evidence of artifact formation in the final formulation. The chemical structures of the characteristic major compounds isolated from the plants used as ingredients are shown in Fig. 1.

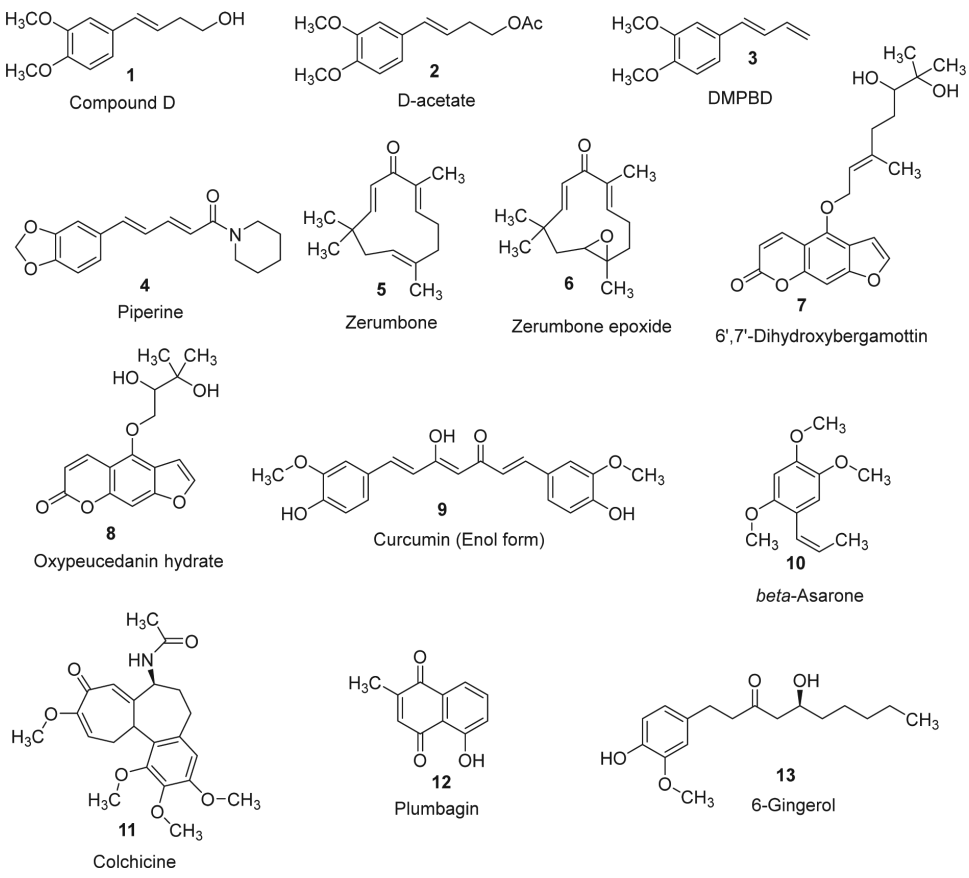

Fig. 1 Chemical structures of major representative compounds from plant ingredients in Ya-Pok-Dud-Pid formulation

The UHPLC chromatogram showing an overview of the phytochemical profile of Ya-Pok-Dud-Pid formulation is presented in Fig. 2, while the TLC analysis is presented in Figs. 4–5 together with the herbal ingredients. Identification was based on using co-chromatography with the authentic standard. *Z. cassumunar* (track 2 in Fig. 3) which was a major ingredient presented in the major bands corresponding to the Ya-Pok-Dud-Pid formulation (track 1 in Fig. 3) and had abundant peaks at retention times of 16.1 min, 27.4 min and 28.6 min in the UHPLC chromatogram, leading to the isolation of the major phenylbutanoids: (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**) and (*E*)-1-(3',4'-dimethoxyphenyl) butadiene (DMPBD, **3**). The identified phenylbutanoids was consistent with other reported results (Seaho et al, 2024; Seaho et al., 2025). Piperine (**4**) was isolated with an abundant peak at a retention time of 34 min in the UHPLC chromatogram (Fig. 2) and had a notable band in the Ya-Pok-Dud-Pid formulation (track 1 in Figure 4). It is a major component in *P. nigrum* and *P. retrofractum*. Zerumbone (**5**) and zerumbone epoxide (**6**) were isolated from *Z. zerumbet*. However, due to the small amount of zerumbone epoxide (**6**), only zerumbone (**5**) could be detected in the Ya-Pok-Dud-Pid formulation. 6',7'-Dihydroxybergamottin (**7**) and oxypeucedanin hydrate (**8**) were isolated from the *C. hystrix* peel. They each had a small peak in the UHPLC chromatogram and a very thin band in the TLC formulation. Curcumin (**9**) was detected in *C. aromatica* but could not be detected in *Z. cassumunar*. The curcumin (**9**) peak from UHPLC overlapped with an unidentified compound from *Z. cassumunar* at a retention time of 48.4 min. Therefore, it could not be analyzed by this method. Identification was also carried out using co-chromatography with authentic standards and β-asarone (**10**) was detected in *Acorus calamus* as a major component. However, the UHPLC peak of β-asarone (**10**) overlapped with oxypeucedanin hydrate (**8**) and had a very thin band in the TLC formulation. Colchicine (**11**) was detected in *Gloriosa superba* and had a small peak at a retention time of 18.1 min in the UHPLC chromatogram and thin band in the TLC formulation Plumbagin (**12**) was detected in *Plumbago indica* and in the Ya-Pok-Dud-Pid formulation at a retention time of 21.1 min in the UHPLC chromatogram. A trace amount of 6-gingerol (**13**) was detected in *Z. officinale* and in the Ya-Pok-Dud-Pid formulation at a retention time of 28.0 min in the UHPLC chromatogram.

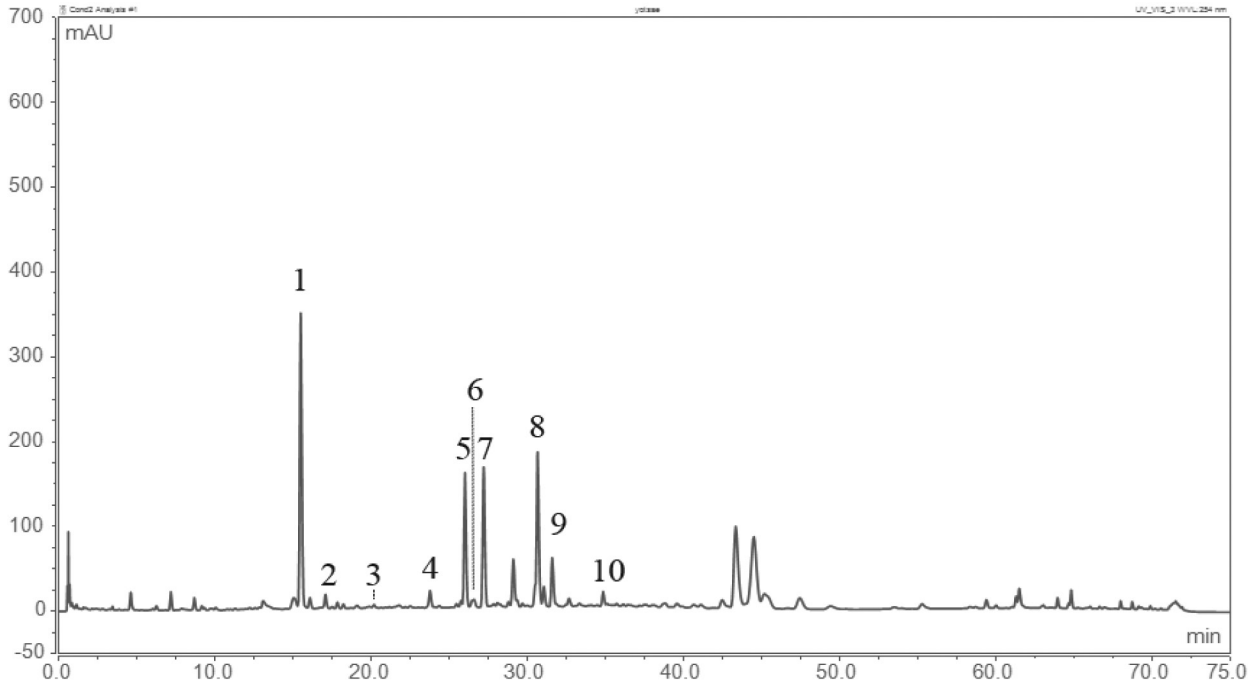

Fig. 2 Ultra-high performance liquid chromatogram of Ya-Pok-Dud-Pid formulation detected at 254 nm ultraviolet wavelength, where Peak 1 = (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), Peak 2 = colchicine (**8**), Peak 3 =plumbagin (**12**), Peak 4=oxypeucedanin hydrate (**8**) overlapped with β-asarone (**10**), Peak 5 = (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**), Peak 6 = 6-gingerol (**13**), Peak 7 = (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**), Peak 8 = piperine (**4**), Peak 9 = zerumbone (**5**) and Peak 10 = 6',7'-dihydroxybergamottin (**7**)

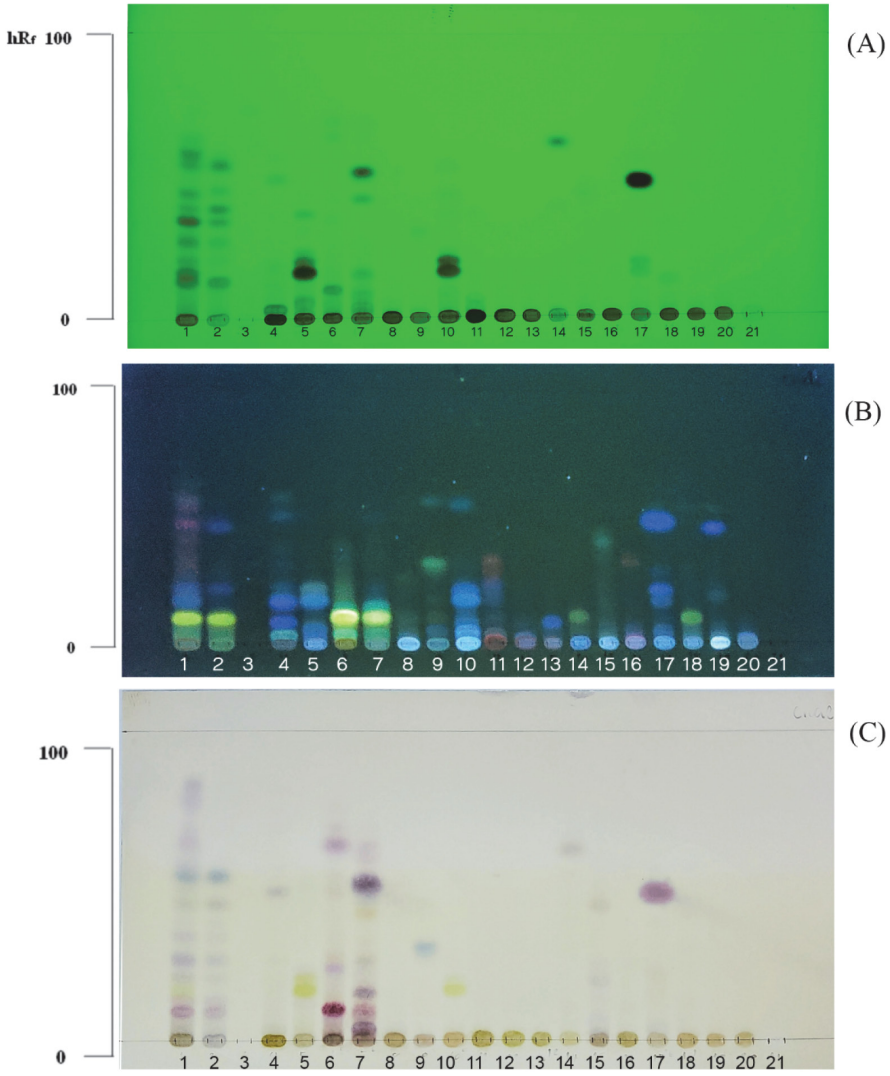

Fig. 3 Thin-layer chromatogram (TLC) of Ya-Pok-Dud-Pid formulation and its herbal ingredients: (A) detected at 254 nm ultraviolet (UV) wavelength, (B) detected under UV 366 nm; (C) sprayed with anisaldehyde-sulfuric reagent and detected under white light, with TLC: silica gel GF_{254}, mobile phase: *n*-hexane:ethyl acetate (7:3, volume per volume), Track 1: Ya-Pok-Dud-Pid formulation, track 2: *Zingiber cassumunar* rhizomes extract, track 3: Camphor, track 4: *Citrus hystrix* fruit peels extract, track 5: *Piper nigrum* fruits extract, track 6: *Curcuma aromatica* roots extract, track 7: *Globba variabilis* rhizomes extract, track 8: *Alpinia galanga* rhizomes extract, track 9: *Plumbago indica* roots extract, track 10: *Piper retrofractum* fruits extract, track 11: *Putranjiva roxburghii* leaves extract, track 12: *Tamarindus indica* leaves extract, track 13: *Senegalia pennata* leaves extract, track 14: *Zingiber zerumbet* rhizomes extract, track 15: *Zingiber officinale* rhizomes extract, track 16: *Cleome viscosa* leaves extract, track 17: *Acorus calamus* roots extract, track 18: *Gloriosa superba* roots extract, track 19: *Crinum asiaticum* leaves extract, track 20: *Tradescantia zebrina* leaves extract and track 21: salt

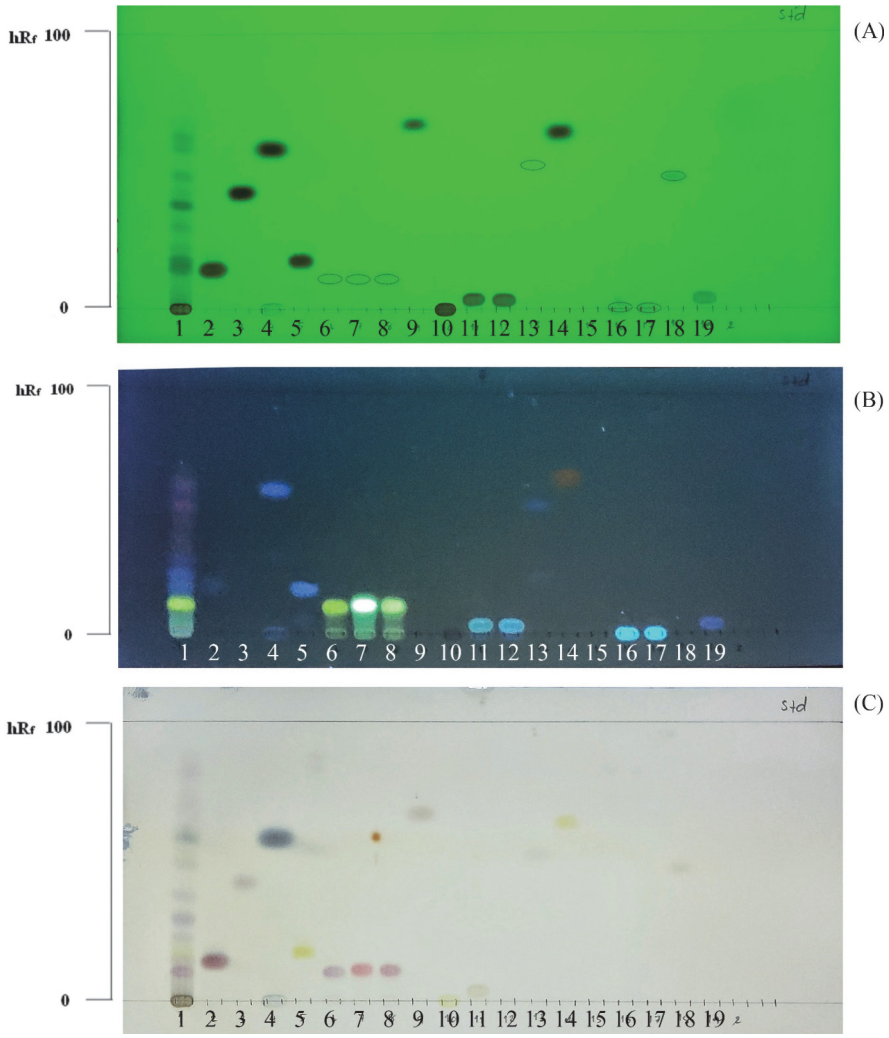

Fig. 4 Thin-layer chromatogram of Ya-Pok-Dud-Pid formulation compared with authentic compounds: (A) detected under at wavelength (UV) 254 nm; (B) detected at UV 366 nm; (C) sprayed with anisaldehyde-sulfuric reagent and detected under white light, whereTLC: silica gel GF_{254}, mobile phase: n-hexane:ethyl acetate (7:3, volume per volume), track 1: Ya-Pok-Dud-Pid formulation, track 2: (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), track 3: (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**), track 4: (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**), track 5: piperine (**4**), track 6: curcumin (**9**), track 7: desmethoxycurcumin, track 8: bismethoxycurcumin, track 9: zerumbone (**5**), track 10: colchicine (**11**), track 11: 6',7'-dihydroxybergamottin (**7**), track 12: oxypeucedanin hydrate (**8**), track 13: β-asarone (**10**), track 14: plumbagin (**12**), track 15: camphor, track 16: lycorine hydrochloride, track 17: 6-gingerol (**13**), track 18: 6-shogaol and track 19: alpinetin

The Ya-Pok-Dud-Pid herbal formulation and its herbal ingredients were extracted using 40% ethanolic extract to investigate the inhibition of nitric oxide production in LPS-induced murine macrophage cell line as *in vitro* anti-inflammatory properties,. The yields of the dried crude extracts are presented in Table 1. The crude extracts were tested for their cytotoxic activity and inhibition of nitric oxide production activity using the RAW 264.7 cell line and these results are presented in Table 1. The Ya-Pok-Dud-Pid extract was anti-inflammatory based on inhibition of nitric oxide production, with an IC_{50} value of 59.86 µg/mL. The crude 40% ethanolic extract of *Z. zerumbet* had the highest activity (IC_{50} 15.45 µg/mL) followed by *C. asiaticum*, *C. aromatica*, *Z. officinale, Z. cassumunar and G. variabilis* (IC_{50} values of 42.80 µg/mL, 63.33 µg/mL, 70.81 µg/mL, 84.22 µg/mL and 92.67 µg/mL, respectively). L-NMMA was used as the positive control and had inhibitory nitric oxide production activity with an IC_{50} value of 17.16 µg/mL (69.12 µM). Of the isolated compounds, zerumbone (**5**) had the highest activity followed by plumbagin (**12**), curcumin (**9**), zerumbone epoxide (**6**), colchicine (**11**), (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**), piperine (**4**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl

acetate (D-acetate, **2**) and (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**) with IC_{50} values of 0.88 µg/mL (4.03 µM), 0.89 µg/mL (4.73 µM), 2.85 µg/mL (7.74 µM), 2.93 µg/mL (12.50 µM), 14.94 µg/mL (37.40 µM), 28.37 µg/mL (149.13 µM), 47.46 µg/mL (166.32 µM), 67.57 µg/mL (269.97 µM) and 78.14 µg/mL (375.20 µM), respectively. Camphor did not produce any inhibitory nitric oxide production activity (IC_{50} > 100 µg/mL). Based on the results of the MTT cell viability assay, the inhibitory effect of nitric oxide production was not due to cell damage.

Table 1 Yield and inhibitory activity of 40% ethanolic extract of Ya-Pok-Dud-Pid formulation and its herbal ingredients on lipopolysaccharide-induced nitric oxide production in RAW 264.7 cell line.

Number	Sample	% Weight per weight in formulation	Yield of 40% ethanolic extract (% weight per weight)[a]	Inhibition of nitric oxide production IC_{50} (µg/mL)[a]
1	Ya-Pok-Dud-Pid formulation	-	18.36 ± 0.63	59.86 ± 8.67
2	*Zingiber cassumunar* rhizomes	30.30	8.92 ± 0.18	84.22 ± 8.04
3	Camphor	15.15	-	>100
4	*Citrus hystrix* fruit peels	3.03	26.73 ± 3.22	> 100
5	*Piper nigrum* fruits	3.03	7.40 ± 0.74	> 100
6	*Curcuma aromatica* roots	3.03	7.82 ± 0.18	63.33 ± 5.90
7	*Globba variabilis* rhizomes	3.03	9.11 ± 0.67	92.67 ± 4.65
8	*Alpinia galanga* rhizomes	3.03	12.87 ± 3.29	> 100
9	*Plumbago indica* roots	3.03	8.33 ± 0.92	> 100
10	*Piper retrofractum* fruits	3.03	15.40 ± 2.69	>100
11	*Putranjiva roxburghii* leaves	3.03	13.74 ± 3.70	> 100
12	*Tamarindus indica* leaves	3.03	15.83 ± 1.88	> 100
13	*Senegalia pennata* leaves	3.03	21.66 ± 1.11	> 100
14	*Zingiber zerumbet* rhizomes	3.03	12.50 ± 0.11	15.45 ± 2.07
15	*Zingiber officinale* rhizomes	3.03	9.57 ± 1.22	70.81 ± 7.23
16	*Cleome viscosa* leaves	3.03	8.19 ± 0.16	> 100
17	*Acorus calamus* roots	3.03	8.18 ± 0.27	> 100
18	*Gloriosa superba* roots	3.03	14.79 ± 2.52	> 6.25
19	*Crinum asiaticum* leaves	3.03	16.25 ± 3.67	42.80 ± 3.61
20	*Tradescantia zebrina* leaves	3.03	16.02[b]	> 100
21	Salt (sodium chloride)	3.03	-	> 100

[a] Expressed as mean ± SD, n = 3; [b] n = 1.

Table 2 Inhibitory activity of isolated compounds on lipopolysaccharide-induced nitric oxide production in RAW 264.7 cell line based on half maximal inhibitory concentration (IC_{50}).

Test sample	Molecular weight (g/mol)	Inhibition of nitric oxide production[a]	
		IC_{50} (µg/mL)	IC_{50} (µM)
L-NMMA (positive control)	248.28	17.16 ± 2.41	69.12 ± 9.71
(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**)	208.26	78.14 ± 10.70	375.20 ± 51.38
(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**)	250.29	67.57 ± 10.47	269.97 ± 41.83
(*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**)	190.24	28.37 ± 11.25	149.13 ± 59.14
Piperine (**4**)	285.35	47.46[b]	166.32[b]
Zerumbone (**5**)	218.33	0.88 ± 0.17	4.03 ± 0.78
Zerumbone epoxide (**6**)	234.33	2.93 ± 0.49	12.50 ± 2.09
Camphor	152.23	> 100	> 656.90
Curcumin (**9**)	368.38	2.85 ± 0.51	7.74 ± 1.38
Colchicine (**11**)	399.44	14.94[b]	37.40[b]
Plumbagin (**12**)	188.18	0.89 ± 0.27	4.73 ± 1.43

[a] Expressed as mean ± SD, n = 3; [b] n = 1.

Discussion

The Thai traditional formulation for the knee poultice called Ya-Pok-Dud-Pid comprised 20 herbal ingredients, with the major constituents being *Z. cassumunar* (30%) and camphor (15%). Its 40% ethanolic tincture is used to treat knee osteoarthritis. Despite its pronounced activity to reduce knee pain clinically, the quality of drug is a crucial concern and its formulation development is an interesting issue. The current study undertook extensive phytochemical study of this drug to characterize the appropriate components for use as chemical markers in quality control.

Investigation of phytochemical composition of Ya-Pok-Dud-Pid formulation revealed its major components cover all major peaks in UHPLC chromatogram (Fig. 2): (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**), (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**), piperine (**4**), zerumbone (**5**), zerumbone epoxide (**6**), 6',7'-dihydroxybergamottin (**7**), oxypeucedanin hydrate (**8**), curcumin (**9**), β-asarone (**10**), colchicine (**11**), plumbagin (**12**) and 6-gingerol (**13**). The TLC chromatogram confirmed the major components and indicated the corresponding spots for its herbal ingredient (Fig. 4). During the measurement the chromatographic profile was unchanged and no artifact was identified in the studied Ya-Pok-Dud-Pid formulation.

Normally, plant species identification involves botanical experts interpreting a plant's morphological characteristics. However, there are some limitations to this approach as it is labor intensive and samples may be presented without required flowers or fruits. Therefore, chemical fingerprinting could provide valuable data for plant identification. The current study provided both UHPLC-DAD and TLC chromatographic chemical fingerprinting together with the characteristic major components of this drug for quality assessment.

Inhibition of nitric oxide production, which is an important inflammatory mediator in the pathogenesis of osteoarthritis (Krasnokutsky et al., 2008), was investigated in the current study using LPS-induced murine macrophage RAW 264.7 cells. The Ya-Pok-Dud-Pid showed iNOS inhibition with an IC_{50} value of 59.86 µg/mL, while L-NMMA, which was used as a positive control had an IC_{50} value of 17.16 µg/mL. The levels of inhibition of the nitric oxide production activity of *Z. zerumbet, C. asiaticum*, *C. aromatica*, *Z. officinale, Z. cassumunar and G. variabilis* appeared to be consistent with Anuthakoengkun and Itharat (2014). In the current study, all the tested compounds (except camphor) had inhibitory effects on nitric oxide production, of which zerumbone (**5**) had the highest activity. Notably, 6',7'-dihydroxybergamottin (**7**) and oxypeucedanin hydrate (**8**) were not tested for inhibition of nitric oxide production activity since crude the *C. hystrix* peel extracts did not show activity.

Abundant UHPLC peaks and TLC spots of the Ya-Pok-Dud-Pid formulation were identified as (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**), (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**) and piperine (**4**). These originated from *Z. cassumunar, P. nigrum and P. retrofractum* and have been proven for their anti-inflammatory properties via different mechanisms. For example, phenylbutenoids—(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**) and (*E*)-1-(3',4'-dimethoxyphenyl) butadiene (DMPBD, **3**)—produced COX-2 inhibition in LPS-induced RAW 264.7 cells (Han et al., 2005). Furthermore, piperine (**4**) inhibited the NF-κB inflammation pathway, leading to the downregulation of pro-inflammatory proteins such as interleukin cytokines (IL-1β, IL-6, IL-8, IL-10) and enzymes (MMP-2, MMP-9) (Dong et al., 2015; Povinelli et al., 2022).

Minor identified components in the Ya-Pok-Dud-Pid formulation were colchicine (**11**), plumbagin (**12**), oxypeucedanin hydrate (**8**), β-asarone (**10**), 6-gingerol (**13**), zerumbone (**5**) and 6',7'-dihydroxybergamottin (**7**). Colchicine (**11**) has been reported to reduce knee pain in osteoarthritic patients and has a wide range of anti-inflammatory activities (Lueng et al., 2015). Plumbagin (**12**) helped in osteoarthritis management by decreasing ROS levels and lipid peroxidation (Petrocelli et al., 2023). In addition, it had an anti-inflammatory effect against chondrocyte-induced inflammation by down-regulating COX-2, iNOS and pro-inflammatory cytokine expression levels (TNF-a, IL-6 and IL-8) (Guo et al., 2017). Furthermore, 6-gingerol (**13**) was reported to inhibit IL-1-induced osteoclast differentiation via suppression of RANKL expression in osteoblasts through reduction of PGE_2 levels (Hwang et al., 2018). Notably, zerumbone (**5**) showed potent iNOS inhibition in the current study and also inhibited MMP-13 and COX-2 expressions of IL-1β-stimulated primary rat chondrocytes (Chien et al., 2016). Oxypeucedanin hydrate (**8**) could reduce the mRNA expression levels of LPS-induced inflammatory factors in RAW 264.7 cells such as TNF-a, IL-6 and IL-1β (Liu et al., 2024).

Camphor, which made up 15% in the studied Ya-Pok-Dud-Pid formulation, was not detected using either the UHPLC or TLC technique and had no inhibitory nitric oxide production activity or other anti-inflammatory properties related to osteoarthritic effects. However, clinical evidence has shown that camphor was effective in reducing the pain from osteoarthritis of the knee (Cohen et al., 2003). It could affect several types of receptors, including heat-sensitive TRPV1, cold-sensitive TRP-M8 and heat-sensitive TRPV3, as well as inhibiting TRPA1 (Duda-Madej et al., 2024). Another trace constituent in the current study was β-asarone (**10**) from *A. calamus* rhizomes, which might be regarded as providing a suitable aroma with some minor benefits to patients with osteoarthritis.

In vitro skin permeation has been reported for (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**) (Wichitchan et al., 2012; Suksaeree et al., 2015), piperine (Asasutjarit et al., 2020), zerumbone (**5**) (Huanbutta et al., 2022), colchicine (**11**) (Abdulbaqi et al., 2018; Ponte et al., 2023) and 6-gingerol (**13**) (Minghetti et al., 2007). Thus, these compounds could be active components that could be benefit knee osteoarthritis in patients. In addition, piperine could enhance the skin permeation of other compounds, such as curcumin (Jantarat et al., 2018).

Thai traditional Ya-Pok-Dud-Pid formulation consists of a complex mixture of various herbal ingredients, including active components, auxiliary components, aromatics, coloring and other inactive components. The pharmacological effects of this poultice might be derived from multiple components due to synergistic effects. Quality control could be applied based on the both amounts of major active chemical markers, such as (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**), (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**) and piperine (**4**), as well as based on its comprehensive chemical fingerprint based on TLC or UHPLC methods, or both. The current study should help in further understanding its biological mechanisms and provide useful information for quality assessment.

The current study identified (*E*)-4-(3',4'-dimethoxyphenyl) but-3-en-1-ol (compound D, **1**), (*E*)-4-(3',4'-dimethoxyphenyl) but-3-en-1-yl acetate (D-acetate, **2**) and (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**) from *Zingiber cassumunar*; piperine from *P. nigrum* and *P.* retrofractum; plumbagin (**12**) from *Plumbago indica*; zerumbone (**5**) and zerumbone epoxide (**6**) from *Zingiber zerumbet*; β-asarone (**10**) from *Acorus calamus*; colchicine (**11**) from *Gloriosa superba*; 6',7'-dihydroxybergamottin (**7**) and oxypeucedanin hydrate (**8**) from *Citrus hystrix* fruit peel; 6-gingerol (**13**) from *Zingiber officinale*. These identified compounds covered the major and active components of the Ya-Pok-Dud-Pid formulation and could be applied as chemical markers in quality assessment. The concentrations of (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), colchicine (**11**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**), (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**), piperine (**4**) and zerumbone (**5**) of different batches of drugs (n = 4) were 224 ± 130 μg/mL, 10.7 ± 4.5 μg/mL, 105 ± 56 μg/mL, 110 ± 88 μg/mL, 191 ± 51 μg/mL and 41 ± 39 μg/mL, respectively. Many active components produced inhibition of nitric oxide production, which is an important inflammatory mediator in the pathogenesis of osteoarthritis. Quality control of the Ya-Pok-Dud-Pid formulation could be carried out based on both the amounts of major active chemical markers—(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D, **1**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate, **2**), (*E*)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD, **3**) and piperine (**4**)—and its comprehensive chemical fingerprint based on TLC or UHPLC methods, or both.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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