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Exploring in vitro anti-proliferative and anti-inflammatory activities of Prasachandaeng remedy, and its bioactive compounds

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Abstract

Background: Prasachandaeng (PSD) remedy has been empirically used in Thai traditional medicine to treat fever in bile duct and liver and cancer patients through Thai folk doctors. However, there have been no scientific reports on the bioactive compounds and bioactivities related to inflammation-associated carcinogenesis or cytotoxicity against cancer cell lines. In this study, we investigated the chemical content of the remedy, and evaluated its cytotoxic activity against two cancer cell lines in comparison with a non-cancerous cell line and determined tumor necrosis factor-alpha (TNF- α) production in a murine macrophage cell line (RAW 264.7) to evaluate anti-inflammatory activity. A novel HPLC method was used for quality control of its chemical content.

Methods: Pure compounds from the EtOH extract of *D. cochinchinensis* were isolated using bioassay-guided fractionation and chemical content of the PSD remedy was determined using HPLC. The cytotoxic activity against the hepatocarcinoma cell line (HepG2) and cholangiocarcinoma cell line (KKU-M156), in comparison with non-cancerous cell line (HaCaT), were investigated using antiproliferative assay (SRB). The anti-inflammatory activity measured by TNF- α production in RAW 264.7 was determined using ELISA.

Results: All crude extracts and isolated compounds exhibited significant differences from vincristine sulfate ($^{****}p < 0.0001$) in their cytotoxic activity against HepG2, KKU-M156, and HaCaT. The PSD remedy exhibited cytotoxic activity against HepG2 and KKU-M156 with IC_{50} values of 10.45 ± 1.98 (SI = 5.3) and 4.53 ± 0.74 (SI = 12.2) $\mu\text{g}/\text{mL}$, respectively. Some constituents from *C. sappan*, *D. cochinchinensis*, *M. siamensis*, and *M. fragrans* also exhibited cytotoxic activity against HepG2 and KKU-M156, with IC_{50} values less than 10 $\mu\text{g}/\text{mL}$. The isolated compounds, i.e., Loureirin B (1), 4-Hydroxy-2,4'-dimethoxydihydrochalcone (2), and Eucomol (3) exhibited moderate cytotoxicity against two cancer cell lines. None of the crude extracts and isolated compounds showed cytotoxicity against HaCaT. *D. cochinchinensis* and PSD remedy exhibited higher anti-inflammatory activity measured as TNF- α production than acetaminophen.

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Conclusion: The findings provide evidence of bioactivity for EtOH extracts of PSD remedy and the isolated compounds of *D. Cochinchinensis*. The results consistent the use clinical activity and use of PSD remedy as a antipyretic treatment for liver and bile duct cancer patients by Thai traditional practitioners.

Keywords: Thai traditional medicine, Cytotoxic activity, Anti-inflammatory, Tumor necrosis factor- α , Bioactive compounds

Background

In Thailand’s contemporary society, the incidence of primary hepatocellular carcinoma is very high. The occurrence of cancer of the liver and bile duct ranks the highest among male patients (19.5%) and seventh among females (3.8%) [1]. Furthermore, hepatocellular carcinoma is the third leading cause of death world-wide [2]. The percentage of hepatocellular carcinoma and cholangiocarcinoma varies greatly between different regions of Thailand. The frequency rate of new cases of cholangiocarcinoma in the Northeast region has increased to the highest in the world [3]. The major risk factors are chronic infections of the hepatitis B virus (HBV), hepatitis C virus (HCV), and high exposure to aflatoxins [4]. Traditionally, cancer patients in Thailand have used complementary medicine for the treatment of chronic diseases, such as degenerative disease, inflammatory, and pain disorders as well as cancers [5].

The current study involves an anti-cancer proliferative activity that has been used for screening the cytotoxicity of herbal medicine extracts and bioactive compounds. The SRB assay is related to the mechanism of antiproliferation of cancer cells [6]. Tumor necrosis factor- α (TNF- α) is a pro-inflammatory cytokine that can be secreted by inflammatory cells, which involves inflammation-associated carcinogenesis. The cytotoxic activity and anti-inflammatory activity through inhibition of TNF- α production is a means of evaluating cancer prevention and therapy [7].

The National List of Essential Medicine (NLEM) of Thailand lists many herbal combinations for treating several diseases, such as headache, dyspepsia, gastritis, fever, etc. Prasachandaeng (PSD) remedy, is an antipyretic medicine used to treat toxic or chronic fever in both adults and children [8]. PSD remedy has been used by Thai folk doctors to treat toxic (chronic) fever in liver and bile duct cancer patients. Thai traditional wisdom describes toxic or chronic fever as the origin of cancer. This ethnotraditional description agrees with modern Western medicine where inflammation or continual fever can lead to cancer [2]. A Thai traditional textbook, Ka-Sai scripture, has a chapter on chronic and cancer symptoms that explains degenerative diseases which lead to chronic disease such as cancer [9]. The causes of chronic diseases, according to Thai traditional medicine (TTM), include

food poisoning, behavioral disorders, chronic inflammation, and infection. Diseases related to hepatocellular carcinoma and cholangiocarcinoma recorded in the Thai traditional scripture, Ka-Sai, and scientific evidence of utility of Thai traditional remedies are consistent with several symptoms described in Western medicine, such as weight loss, anemia, pale skin, abdominal pain, lack of appetite, insomnia, yellow urine, chronic fever, constipation, and unhealthy body and mind (Table 1). However, the PSD remedy has not been scientifically investigated with respect to bioactive compounds and bioactivities related to inflammation-associated carcinogenesis. Therefore, the aims of this study were to isolate compounds using a bioassay-guided fractionation from the main ingredients of PSD remedy using HPLC analysis for quality control. and to investigate cytotoxicity activity against two cancer cell lines, i.e., hepatocarcinoma (HepG2) and cholangiocarcinoma (KKU-M156) in comparison with a non-cancerous cell line (HaCaT) using the antiproliferative assay (SRB assay). The anti-inflammatory

Table 1 The comparison of symptoms of liver and bile duct cancer in Thai traditional medicine (TTM) and modern medicine

Symptoms	Thai traditional medicine ^a	Modern medicine ^b
Anemia	✓	-
Pale skin	✓	-
Insomnia	✓	-
Constipation	✓	-
Chronic fatigue	✓	-
Jaundice	✓	✓
Itching	-	✓
Light-colored	✓	✓
Greasy stools	-	✓
Dark urine	-	✓
Abdominal pain	✓	✓
Loss of appetite	✓	✓
Weight loss	✓	✓
Nausea and vomiting	✓	✓
Chronic fever	✓	✓

(✓) indicates found as a symptom, (-) indicates not found as a symptom

^a Thai traditional medical textbook, The Rehabilitation Foundation for Thai Traditional Medicine and Ayurved Thamrong School, 2007

^b American cancer society, 2020

activity measured as inhibition of TNF- α production in the murine macrophage cell line (RAW 264.7) was also investigated using an enzyme-linked immunosorbent assay (ELISA).

Methods

Chemicals and reagents

Ethanol (EtOH), 95%, was purchased from C.M.J. Anchor company, Thailand. Acetic acid, Sulfuric acid and Trichloroacetic acid (TCA) were purchased from Merck, Germany. Analytical grade reagents, i.e., hexane, chloroform (CHCl₃), ethyl acetate (EtOAc), methanol (MeOH), dimethylsulfoxide (DMSO), hydrochloric acid (HCl) were purchased from RCI Labscan, Thailand. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), minimum essential medium (MEM), penicillin–streptomycin (P/S), and phosphate-buffered saline (PBS) were purchased from Biochrom, Germany. Sodium bicarbonate (NaHCO₃) was purchased from BHD, United Kingdom. Sodium hydroxide (NaOH) was purchased from Univar, Australia. Sulforhodamine B sodium salt, Tris [hydroxymethyl] aminoethane, HEPES buffer solution, nutrient mixture F-12 Ham (HAM's F12), and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich, USA. Trypan blue stain 0.4% and trypsin–EDTA were purchased from Gibco, USA. Silica Gel 60 (particle size 0.063–0.200 mm) for vacuum liquid chromatography (VLC), Silica Gel 60 (particle size 0.040–0.063 mm) for column chromatography (CC), and thin layer chromatography (TLC) silica gel 60 F254 were purchased from Merck, Germany. Anisaldehyde reagent was purchased from Fluka, Switzerland. NMR spectra were obtained with a Bruker Avance 400 spectrometer at 400 and 500 MHz for ¹H NMR and 100 and 125 MHz for ¹³C NMR. The chemical shifts were recorded in δ_H , δ_C (ppm)

in CDCl₃. Rotary evaporator was purchased from Buchi, Switzerland. UV spectrophotometer was purchased from SHIMADZU, Japan. CO₂ incubator was purchased from Shellab, USA. Laminar flow cabinet was purchased from Boss tech, Thailand. Microplate reader was purchased from Bio Tek instrument, USA.

Identification of plant ingredients of PSD remedy

Plant ingredients of PSD remedy were harvested from several regions of Thailand. Species identification was approved by the Herbarium of Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla, Thailand as shown in Table 2. All plant materials were carried out according to the standard of quality control of plant materials published earlier [10].

Preparation and extraction

Plant ingredients were cleaned, sliced to small pieces, and dried at 45 °C in a hot air oven. The plant ingredients were weighed and mixed according to the PSD remedy proportion as shown in Table 2. The PSD remedy powder (1,000 g) was macerated with EtOH (5,000 mL) for 72 h and filtered through a Whatman filter paper No. 1 and re-macerated twice. The combined extract was dried using a rotary evaporator. Each crude powder of plant (200 g) was extracted with the same method as above. All crude extracts were kept at -20 °C before bioactivities testing and chemical analysis.

Isolation of compounds from *Dracaena cochinchinensis* (Lour.) S.C. Chen

In this study, *Dracaena cochinchinensis* (Lour.) S.C. Chen was the main herbal ingredient of PSD remedy constituting 50% w/w of all proportions and the EtOH extract

Table 2 The general information of the plant ingredients from Prasachandaeng (PSD) remedy

Plant species (Family)	Voucher specimen number	Part used	Collected from	Proportion (%w/w)
<i>Bouea macrophylla</i> Griff. (Anacardiaceae)	SKP 009 02 02 01	Twig	Nakhonnayok	6.25
<i>Caesalpinia sappan</i> L. (Leguminosae)	SKP 098 03 19 01	Heartwood	Sukhothai	6.25
<i>Citrus aurantiifolia</i> (Christm.) Swingle (Rutaceae)	SKP 166 03 01 01	Twig	Nakhonpathom	6.25
<i>Dracaena cochinchinensis</i> (Lour.) S.C. Chen (Dracaenaceae)	SKP 065 04 12 01	Heartwood	Phitsanulok	50.00
<i>Heliciopsis terminalis</i> (Kurz) Sleumer (Proteaceae)	SKP 187 19 18 01	Twig	Chiangmai	6.25
<i>Jasminum sambac</i> (L.) Aiton (Oleaceae)	SKP 129 10 19 01	Flower	Nakhonpathom	1.56
<i>Kaempferia galanga</i> L. (Zingiberaceae)	SKP 206 11 07 01	Rhizome	Nakhonpathom	6.25
<i>Ligusticum chuanxiong</i> Hort. (Umbelliferae)	SKP 199 12 19 01	Rhizome	China	6.25
<i>Mammea siamensis</i> T.Anderson. (Calophyllaceae)	SKP 083 13 19 01	Flower	Chantaburi	1.56
<i>Mesua ferrea</i> L. (Calophyllaceae)	SKP 083 13 06 01	Flower	Phichit	1.56
<i>Myristica fragrans</i> Houtt. (Myristicaceae)	SKP 121 13 06 01	Heartwood	Indonesia	6.25
<i>Nelumbo nucifera</i> Gaertn. (Nelumbonaceae)	SKP 125 14 14 01	Pollen	Nakhonpathom	1.56

of *D. cochinchinensis* (DC95) showed the most potent cytotoxic activity against two cancer cell lines (HepG2 and K KU-M156). Therefore, the DC95 was chosen for the isolation of pure compounds by bioassay-guided fractionation. DC95 (70 g) was chromatographed in a vacuum liquid chromatography (VLC) using silica gel 60 (300 g) with gradient elution which provided five fractions as follows: hexane (Fraction 1: 2,000 mL), hexane: CHCl₃ (Fraction 2: 1:1, 2,000 mL), chloroform (Fraction 3: 2,000 mL), CHCl₃: MeOH (Fraction 4: 1:1, 2,000 mL) and MeOH (Fraction 5: 2,000 mL), respectively. The percentage of yield as %w/w of the starting weight of crude extracts are shown in Table 3. Based on the cytotoxic activity against HepG2 and K KU-M156 cell lines by SRB assay, F3 was chosen for the bioassay-guided isolation as it demonstrated highest cytotoxicity against two types of cancer cell lines (HepG2 and K KU-M156) with the IC₅₀ values of 43.14 ± 1.50 and 42.26 ± 1.07 µg/mL, respectively. F1-5 fractions showed no cytotoxicity against a human normal cell line (HaCaT) with the IC₅₀ > 50 µg/mL. Fraction 3 (7.0 g) was chromatographed in a column chromatography (CC) on silica gel 60 (150 g) with gradient elution to give six fractions as followed: hexane:EtOAc (6:4, 5,000 mL), hexane:EtOAc (7:3, 500 mL), hexane:EtOAc (1:1, 500 mL), EtOAc (500 mL), EtOAc:MeOH (1:1, 500 mL), and MeOH (500 mL), respectively. The eluent was collected and examined by thin layer chromatography (TLC) with UV 254 detector at 356 nm, and was sprayed with anisaldehyde reagent. The structure of the isolated compounds were identified by ¹H-NMR, ¹³C-NMR, DEPT135, DEPT90, COSY, NOESY, HSQC, and HMBC. Subfraction Fr.11 (416 mg)

was also purified by CC on silica gel 60 (50 g) and eluted with hexane: CHCl₃ (1:9) to afford a 2 (8.4 mg, 0.12% w/w of crude extract) as a white amorphous powder and a 3 (10.5 mg, 0.15% w/w of crude extract) as colorless crystal. The subfraction Fr.15 (146 mg) was also purified by recrystallization with hexane: CHCl₃ (7:3) to provide a 1 (6.8 mg, 0.10%w/w of crude extract) as a white amorphous powder.

Determination of the isolated compounds in Prasachandaeng remedy

Instruments

The HPLC method followed a protocol previously described [11, 12] with only a slight modification. It was performed on an Agilent® 1200 HPLC system (Agilent Technologies, USA) composing of a solvent degasser (G1322A), a quaternary solvent pump (G1311A), an autosampler (G1329A), a column oven (G1316A), and a photodiode array detector (G1315D). The chromatographic data were processed by the Chemstation® software revision B.04.01 SP1. The reversed-phase C18 column was Phenomenex® Luna SU C18(2)/100(A), column size 150 × 4.6 mm.

High performance liquid chromatography analysis

The HPLC method was modified from Pipatrattanaseree et al., 2019 [12]. The EtOH extract of PSD remedy was prepared at a concentration of 10 mg/mL. An accurately weighed extract was dissolved with methanol and sonicated for 15 min. The solution of each isolated compound was prepared at a concentration of 1 mg/mL in methanol for the identification of chromatograms and quantitative

Table 3 The IC₅₀ µg/mL ± SEM of cytotoxicity against two cancer cell lines and one non-cancerous cell line of VLC isolated fractions, isolated pure compounds and ethanolic extract of *D.cochinchinensis* and comparison with a standard chemotherapeutic drug (Vincristine sulfate) (n = 3)

Sample	Code	%yield (w/w)	IC ₅₀ µg/mL ± SEM and Selective index (SI)		
			HepG2	K KU-M156	HaCat
Fraction 1 (hexane)	F1	0.19	NT	NT	NT
Fraction 2 (hexane: CHCl ₃ , 1:1)	F2	2.31	> 100****	> 100****	> 100****
Fraction 3 (CHCl ₃)	F3	14.57	43.14 ± 1.50**** (SI = 2.2)	42.26 ± 1.07**** (SI = 2.2)	92.96 ± 2.22****
Fraction 4 (CHCl ₃ : MeOH, 1:1)	F4	78.65	51.95 ± 0.37**** (SI = 1.8)	61.51 ± 5.08**** (SI = 1.5)	95.01 ± 0.42****
Fraction 5 (MeOH)	F5	4.27	> 100****	> 100****	> 100****
Loureirin B (1)	P1	0.10	20.02 ± 0.46**** (SI = 4.3)	21.26 ± 3.17**** (SI = 4.1)	86.12 ± 2.13****
4-Hydroxy-2,4'-dimethoxydihydrochalcone (2)	P2	0.12	20.71 ± 0.49**** (SI = 3.2)	33.21 ± 2.10**** (SI = 2.0)	66.00 ± 0.68****
Eucomol (3)	P3	0.15	25.76 ± 1.56**** (SI = 1.8)	7.12 ± 0.56**** (SI = 6.5)	46.55 ± 0.88****
EtOH of <i>D.cochinchinensis</i>	DC95	10.45	7.72 ± 1.87**** (SI = 5.2)	5.27 ± 5.01**** (SI = 7.7)	40.47 ± 0.39****
Vincristine sulfate	-	-	0.012 ± 0.0005 (SI = 0.00058)	0.0026 ± 0.001 (SI = 0.0026)	0.000007 ± 0.00

NT mean not tested due to weight of extract less than 5 mg. SI Selective index calculated by IC₅₀ of non-cancerous cells / IC₅₀ of cancer cells. Data were presented as mean ± SEM and analyzed by one-way ANOVA and Dunnett's multiple comparison tests. Significant different presented the ****p < 0.0001 compared with a standard drug (Vincristine sulfate) in corresponding cell line

analysis of chemical contents. All samples were filtered through 0.45 micron before analysis with the HPLC system. The serial dilution of three marker compounds of PSD remedy, i.e., Loureirin B (1), 4-Hydroxy-2,4'-dimethoxydihydrochalcone (2), and Eucomol (3) were injected into HPLC, and the calibration curves constructed according to their responses. All standard curves demonstrated linearity with the $r^2 > 0.99$ within the linear range. All quantitatively determined data from the isolated compounds in PSD remedy was expressed as the mean \pm standard error of the means (SEM) of at least three independent experiments.

Chromatographic system

The solvent system consisted of a gradient mobile phase of water (A) and acetonitrile (B) which was programmed as follows: 0–5 min, (A:B; 95%:5%), 5–55 min, (A:B; 30%:70%), and 55–60 min, (A:B; 90%:10%), respectively. The flow rate was set at 1 mL/min and the pressure limit was 400 bars. The samples were injected into the HPLC system and detected with diode array detector at 280 nm wavelength.

$$\% \text{ Inhibition of cell growth} = \left[(\text{Mean of OD}_{\text{control}} - \text{Mean of OD}_{\text{sample}}) \div \text{Mean of OD}_{\text{sample}} \right] \times 100$$

Cytotoxic activity using antiproliferative assay (Sulforhodamine B assay)

Cell culture

Hepatocellular carcinoma (HepG2; ATTC No. HB-8065) was cultured in Minimum Essential Media (MEM) supplemented with 10% heated-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin (P/S). Cholangiocarcinoma cell line (KKU-M156) was cultured in HAM's F12 supplemented with 10% FBS, 1% P/S, and 12.5 mM HEPES. In addition, one non-cancerous human keratinocyte cell line (HaCaT; No. 300493-SF) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% P/S [13].

In vitro Sulforhodamine B assay

This assay followed the previously described protocol [5, 13]. The various concentrations (1, 10, 50, and 100 $\mu\text{g/mL}$) of the crude extracts were investigated against two human cancer cell lines and one non-cancerous cell line. The cell lines were washed with PBS and the cells were detached with 0.025% trypsin–EDTA to make a single cell suspension. A 5 mL medium was then added to the flask to stop the trypsin–EDTA activity. The viable cells were counted by trypan blue exclusion in a haemocytometer. A single-cell suspensions density of HepG2, KKU-M156, and HaCaT were diluted with each medium to give

optimal densities of 2×10^3 , 3×10^3 , and 8×10^3 cells/well, respectively. The 100 μL /well of these cell suspensions were seeded in 96-well plates and incubated at 37 °C with 5% CO₂ at 95% humidity for 24 h. Then, 100 μL of a sample solution was added to each well. The control was the medium mixed with 2% DMSO. The 96-well plates were incubated at 37 °C with 5% CO₂ at 95% humidity for 72 h. The mixture in the well was removed and washed with 200 μL fresh medium. The 96-well plate was further incubated for 72 h, then the cell in 96-well plates was fixed with 40%TCA and washed with water five times. Finally, the fixed cell in the 96-well plate was strained with Sulforhodamine B sodium salt and the percentage of inhibition of cell growth was measured colorimetrically using the SRB assay [5, 13, 14]. The herbal extract and pure compounds were considered to have potent cytotoxicity if the IC₅₀ values were ≤ 20 and ≤ 4 $\mu\text{g/mL}$, respectively [15]. The % inhibition of cell growth was calculated by the equation shown below, and the IC₅₀ values were calculated using the Prism program. The protocol of in vitro Sulforhodamine B assay is shown in Fig. 1.

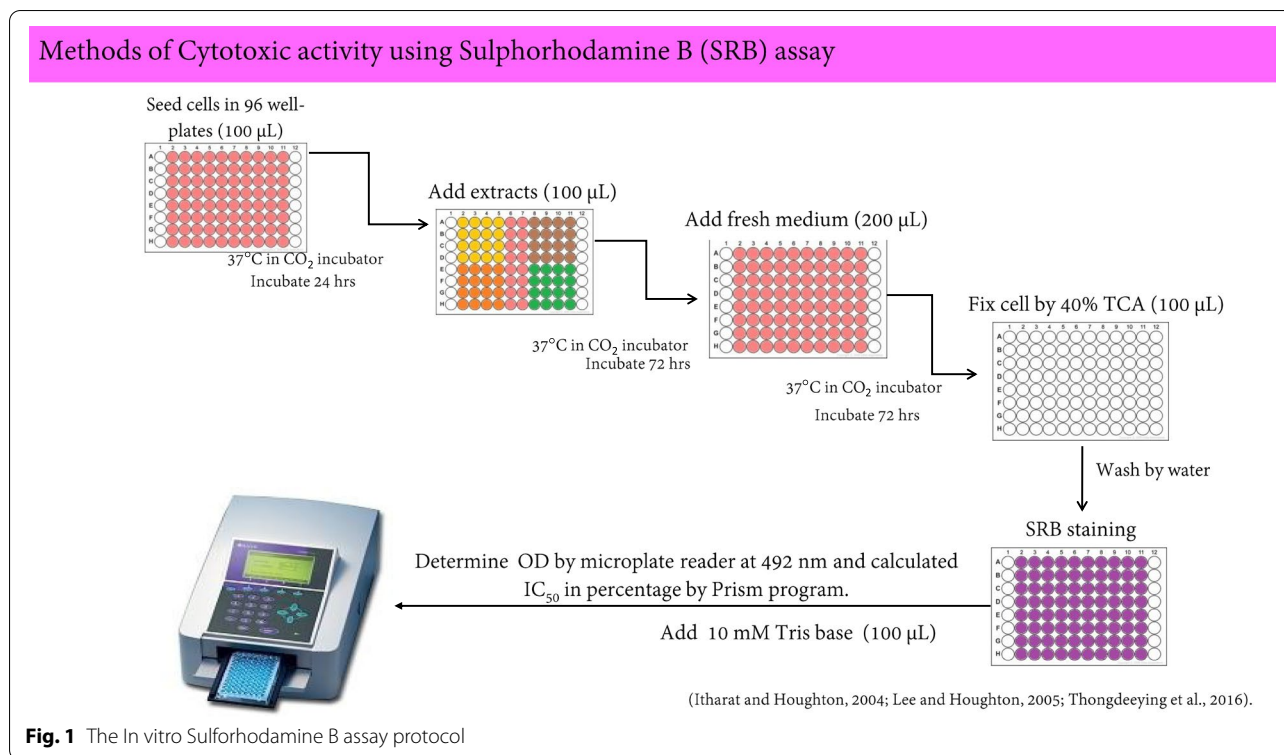
where: OD_{control} = OD of medium with 2% of DMSO and OD_{sample} = OD of crude extract.

The Selectivity index (SI) exhibited the ratio of the half-maximal inhibitory concentration (IC₅₀) of non-cancerous cell line and the half-maximal inhibitory concentration (IC₅₀) of cancer cell line [16]. Additionally, when the SI value was determined to be higher than three it was chosen as a prospective in vitro anti-proliferative sample [17].

$$\text{Selectivity index (SI)} = \text{IC}_{50} \text{ of non-cancerous cell line} \div \text{IC}_{50} \text{ of cancer cell line}$$

Anti-inflammatory activity on inhibition of TNF- α production

The tumor necrosis factor-alpha (TNF- α) is the principal mediator of inflammation in response to gram-negative bacteria. It is mainly produced by LPS-activated mononuclear phagocytes. The TNF- α ELISA can quantify TNF- α in the supernatant of cell culture medium [18]. The assay was carried out using the TNF-alpha ELISA kit (Thermo Fisher® scientific, USA). The murine macrophage cell line (RAW 264.7) was cultured in DMEM medium containing 10% heat-inactivated FBS, 10⁴ $\mu\text{g/mL}$ P/S. Firstly, the viable cells were counted using trypan blue exclusion in a haemocytometer. The single cell suspension of murine macrophage cell line was diluted with



the medium to provide an optimal density of 10⁵. The cell suspension was seeded, in a 100 µL/well, in 96-well plates and incubated at 37 °C with 5% CO₂ atmosphere at 95% humidity for 24 h. Secondly, 100 µL of fresh medium containing 5 ng/mL of lipopolysaccharide and 100 µL at 100 µg/mL of test sample for screening. Besides, if the test sample shows the %inhibition of TNF- α production of more than 50%, we will investigate the various concentrations at 1, 10, 50, and 100 µg/mL of test sample for calculation of IC₅₀. Then, the test sample was added and

µL of TMB development solution was added to each well and incubated for 10 min in the dark on a plate shaker set to 400 rpm and 100 µL of stop solution was added to each well. The 96-well plate was shaken for 1 min and then incubated for 20 min. The concentrations of TNF- α in the wells were measured with a microplate reader at 450 nm [19, 20]. The % inhibition of TNF-α production was calculated using the equation below, and the IC₅₀ values were calculated using the Prism program. The protocol of the anti-TNF- α production is shown in Fig. 2.

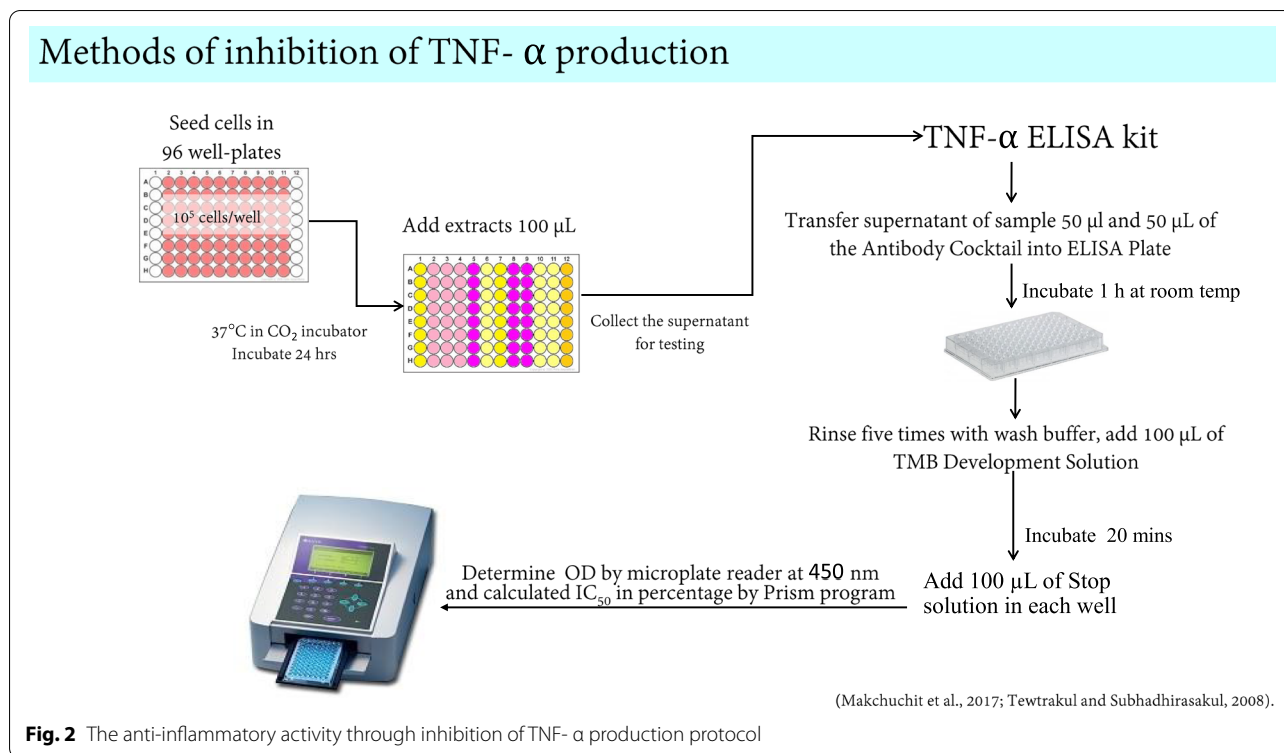
$$\%Inhibition = \left[\frac{(\text{Mean of } OD_{\text{control}} - \text{Mean of } OD_{\text{sample}})}{\text{Mean of } OD_{\text{control}}} \right] 100$$

incubated for 24 h. The control included 2% DMSO solution mixed with the medium, in place of the test samples in the analyses. This protocol was followed according to TNF-α ELISA kit (Thermo Fisher® scientific, USA). The various reagents, such as biotinylated detection antibody, streptavidin-HRP, HRP diluent, wash buffer, chromogen stop solution, were prepared before starting the experiment. Firstly, 50 µL of all samples or standard were added to appropriate wells. Secondly, 50 µL of the antibody cocktail was added to each well and the plate sealed and incubated for 1 h at room temperature on a plate shaker. Then, the wells were washed with wash buffer. Lastly, 100

Where: Mean of OD_{control} = Mean of OD_{control (-LPS)} - Mean of OD_{control (+LPS)}, Mean of OD_{sample} = Mean of OD_{sample (+LPS)} - Mean of OD_{control (+LPS)}.

Statistical analysis

All data are expressed as the mean ± standard error of the means (SEM) of at least three independent experiments. The % inhibition values were calculated using the Microsoft Excel program. The IC₅₀ values and statistical significance were calculated using the Graph-Pad Prism software, version 8.0.1 (San Diego, CA, USA). Statistical differences were analyzed by one-way



ANOVA, followed by Dunnett's multiple comparison tests using. The statistical significance was assessed at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Results

Structure elucidation of isolated pure compound and their bioactivity

The results of the percentage of yield (%w/w) and cytotoxic activity are shown in Table 3. Fraction 3 (F3) was chosen for the bioassay-guided fractionation as it exhibited potent cytotoxicity against two cancer cell lines (HepG2 and KKV-M156) in comparison with human non-cancerous cell line (HaCaT) with the IC_{50} value of 43.14 ± 1.50 (SI=2.2) and 42.26 ± 1.07 (SI=2.2) $\mu\text{g/mL}$, respectively. F2, F4, and F5 did not show cytotoxic activity against two cancer cell lines and one type of non-cancerous cell line. Three isolated compounds from the EtOH extract of *D. cochinchinensis*, were Loureirin B (1), 4-Hydroxy-2,4'-dimethoxydihydrochalcone (2), and Eucomol (3). The ^1H and ^{13}C NMR spectral data of compound 2 were closely related to compound 1 (Table 4), except the methoxy group at C-6 was absent.

Loureirin B (1) was obtained as a white amorphous powder. The ^1H and ^{13}C data were recorded at 500 and 125 MHz in CDCl_3 , respectively. The NMR spectrum of

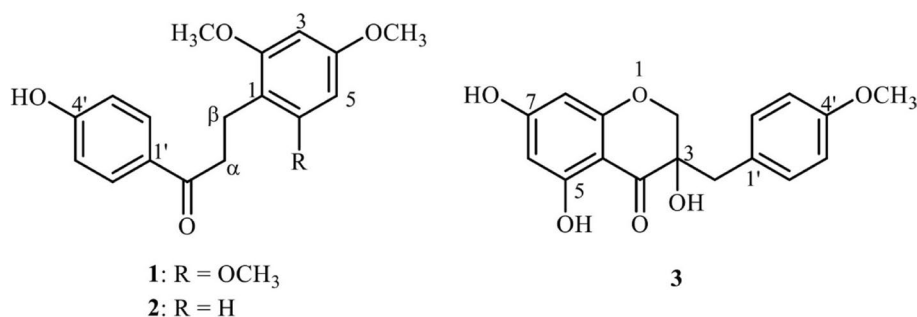
compound 1 displayed the presence of *para*-disubstituted aromatic protons at δ 7.92 and 6.87 (each d, $J=8.7$ Hz, H-2', H-6' and H-3', H-5', respectively) and doublet *meta* proton at δ 6.12 (d, $J=2.3$ Hz, H-3, and H-5). Three methyl protons at δ 3.80 (6H, s) and 3.76 (3H, s) suggest the presence of methoxy groups at 2-OCH₃, 6-OCH₃, and 4-OCH₃, respectively. The appearance of the carbonyl group at δ 200.3 and two aliphatic protons at δ 3.04 (2H, H- α) and 2.97 (2H, H- β) displayed a similar signal pattern to those of retrodihydrochalcone [21]. In the HMBC spectra (Table 4 and Fig. 3), the position of H- α (δ 3.04) shows correlations with C-1 (δ 109.7), C- β (δ 18.6) and C=O (δ 200.3). The aromatic proton H-2' (δ 7.92) correlated with C-1' (δ 129.9), C-3' (δ 115.2), C-4' (δ 160.2), C-6' (δ 130.8) and C=O (δ 200.3), and the aromatic proton H-3 (δ 6.12) correlated with C-1 (δ 109.7), C-4 (δ 159.5), C-5 (δ 90.4) and C- β (δ 18.6). These spectral data identified compound 1 as Loureirin B (1-(4-hydroxyphenyl)-3-(2,4,6-trimethoxyphenyl)propan-1-one), which structure has been described in a previous report [21]. Compound 1 exhibited potent cytotoxicity against HepG2 and KKV-M146 with the IC_{50} values of 20.02 ± 0.46 and 21.26 ± 3.17 $\mu\text{g/mL}$, respectively (Table 3).

4-Hydroxy-2,4'-dimethoxydihydrochalcone (2): White amorphous powder; ^1H and ^{13}C data were recorded at 400 and 100 MHz in CDCl_3 , respectively. The ^1H and ^{13}C NMR spectral data of compound 2 were closely related to

Table 4 NMR spectral data of compounds 1 and 2 in CDCl₃

Position	1			2		
	δ_C	δ_H (J in Hz)	HMBC ($\delta_H \rightarrow \delta_C$)	δ_C	δ_H (J in Hz)	HMBC ($\delta_H \rightarrow \delta_C$)
1	109.7			122.0		
2	158.7			159.5		
3	90.4	6.12 (s)	C-1, C-4, C-5, C- β	98.7	6.44 (d, 2.3)	C-1, C-2, C-5
4	159.5			158.4		
5	90.4	6.12 (s)	C-1, C-3, C-4, C- β	104.1	6.41 (dd, 8.2, 2.3)	C-1, C-3
6	158.7			130.3	7.07 (d, 8.2)	C-2, C-3, C-4, C- β
1'	129.9			130.2		
2'	130.8	7.92 (d, 8.7)	C-1', C-3', C-4', C-6', C=O	130.8	7.91 (d, 8.8)	C-1', C-3', C-4', C-6', C=O
3'	115.2	6.87 (d, 8.7)	C-1', C-2', C-5'	115.3	6.88 (d, 8.8)	C-1', C-4', C-5'
4'	160.2			160.2		
5'	115.2	6.87 (d, 8.7)	C-1', C-3', C-6'	115.3	6.88 (d, 8.8)	C-1', C-3', C-4'
6'	130.8	7.92 (d, 8.7)	C-1', C-2', C-4', C-5', C=O	130.8	7.91 (d, 8.8)	C-1', C-2', C-4', C=O
α	38.4	3.04 (m)	C-1, C- β , C=O	38.9	3.17 (m)	C-1, C- β , C=O
β	18.6	2.97 (m)	C-1, C-6, C- α , C=O	25.4	2.95 (m)	C-1, C-2, C-6, C- α C=O
2-OCH ₃	55.6	3.80 (s)	C-2	55.4	3.78 (s)	C-2
4-OCH ₃	55.3	3.76 (s)	C-4	55.3	3.78 (s)	C-4
6-OCH ₃	55.6	3.80 (s)	C-6			
C=O	200.3	-	-	199.3		

Note: Compound 1; ¹H and ¹³C data were recorded at 500 and 125 MHz and Compound 2; ¹H and ¹³C data were recorded at 400 and 100 MHz

**Fig. 3** The chemical structure of three isolated compounds from the heartwood of *Dracaena cochinchinensis* (Lour.) S.C. Chen

compound 1 (Table 4 and Fig. 3), except for the absence of methoxy group at 6-OCH₃ and the appearance of 1,2,4-trisubstituted aromatic proton at δ 7.07 (1H, d, J =8.2 Hz, H-6), δ 6.44 (1H, d, J =2.3 Hz, H-3), and δ 6.41 (1H, dd, J =8.2, 2.3 Hz, H-5). The location of H-6 was assigned by HMBC spectra (Table 4) in which the aromatic proton H-6 (δ 7.07) correlated with carbon C-2 (δ 159.5), C-3 (δ 98.7), C-4 (δ 158.4) and C- β (δ 18.6). These spectral data confirm compound 2 as 4-Hydroxy-2,4'-dimethoxydihydrochalcone. It provides identical spectral data with those described in a previous report [22].

Eucomol (3): Colorless crystal; ¹H and ¹³C data were recorded at 500 and 125 MHz in CDCl₃, respectively

(Table 5 and Fig. 4). The NMR spectrum of compound 3 showed one methoxyl group at δ 3.80 (s, 4'-OCH₃) and three hydroxyl group at δ 11.29 (s, 5-OH), and 3.40 (s, 3-OH). The para-disubstituted aromatic proton was present at δ 7.13 and 6.86 (each d, J =8.3 Hz, H-2', H-6' and H-3', H-5', respectively), and *meta* proton at δ 6.04 (s, H-8) and 6.01 (s, H-6). The AB system of methylene proton at C-2 appeared at δ 4.19 and 4.07 (d, J =11.1 Hz). In addition, the aliphatic methylene proton at C-9 was shown at δ 2.94 (2H, d, J =7.0 Hz). In the HMBC spectra (Table 5), the methylene proton H-2 (δ 4.19 and 4.07) correlated with C-3 (δ 72.2), C-4 (δ 198.1), C-8a (δ 163.0) and C-9 (δ 40.6),

Table 5 NMR spectral data (500 MHz for ^1H and 125 MHz for ^{13}C) of compound 3

Position	δ_{C}	δ_{H} (J in Hz)	HMBC ($\delta_{\text{H}} \rightarrow \delta_{\text{C}}$)
2	71.7	4.19 (d, 11.1) 4.07 (d, 11.1)	C-3, C-4, C-8a, C-9
3	72.2		
4	198.1		
5	164.1		
6	95.7	6.01 (d, 6.01)	C-4a, C-8
7	165.3		
8	97.0	6.04 (s)	C-4a, C-6, C-7
9	40.6	2.94 (d, 7.0)	C-3, C-4, C-1', C-2'
1'	126.0		
2'	131.5	7.13 (d, 8.3)	C-3', C-4', C-6'
3'	113.7	6.86 (d, 8.3)	C-1', C-4', C-5'
4'	158.8		
5'	113.7	6.86 (d, 8.3)	C-1', C-3', C-4'
6'	131.5	7.13 (d, 8.3)	C-2', C-4', C-5'
4a	100.5		
8a	163.0		
4'-OCH ₃	55.2	3.80 (s)	C-4'
3-OH	-	3.40 (s)	C-2, C-4, C-4a,
5-OH	-	11.29 (s)	C-4, C-4a C-5

the methylene proton H-9 (δ 2.94) correlated with C-3 (δ 72.2), C-4 (δ 198.1), C-1' (δ 126.0) and C-2' (δ 131.5), the aromatic proton H-6 (δ 6.01) correlated with C-4a (δ 100.5) and C-8 (δ 97.0), and the aromatic proton H-2' (δ 7.13) correlated with C-3' (δ 113.7),

C-4' (δ 158.8) and C-6' (δ 131.5). These spectral data identified compound 3 as Eucomol ((3*S*)-3,5,7-trihydroxy-3-[(4-methoxyphenyl)methyl]-2*H*-chromen-4-one, which structure has been described in the literature [23]. Eucomol (3) showed potent cytotoxicity against KKU-M156 and HepG2 with the IC_{50} values of 7.12 ± 0.56 and 25.76 ± 1.56 $\mu\text{g/mL}$, respectively (Table 3). The structure of Eucomol differs from that of the classical isoflavones by the insertion of a carbon atom into the skeleton.

Quantitative determination of the isolated compounds in PSD remedy

The contents of the three isolated compounds, Loureirin B (1), 4-Hydroxy-2,4'-dimethoxydihydrochalcone (2), and Eucomol (3), were simultaneously determined by a HPLC method. The three isolated compounds were the major chemical constituents of PSD95. The contents of each isolated compounds were calculated against its corresponded calibration curve which showed the r^2 value greater than >0.99 . (1) showed the highest content (28.71 ± 1.22 mg/g) followed by (3) (24.81 ± 0.17 mg/g) and (2) (18.67 ± 0.14 mg/g), respectively. The chemical constituent contents and HPLC chromatogram of marker compounds are shown in Fig. 3 and 4.

In vitro cytotoxicity of PSD remedy and plant ingredients

All crude extracts and isolated compounds exhibited significant differences from the anti-cancer drug vincristine sulfate ($^{***}p < 0.0001$) in their cytotoxic activity against

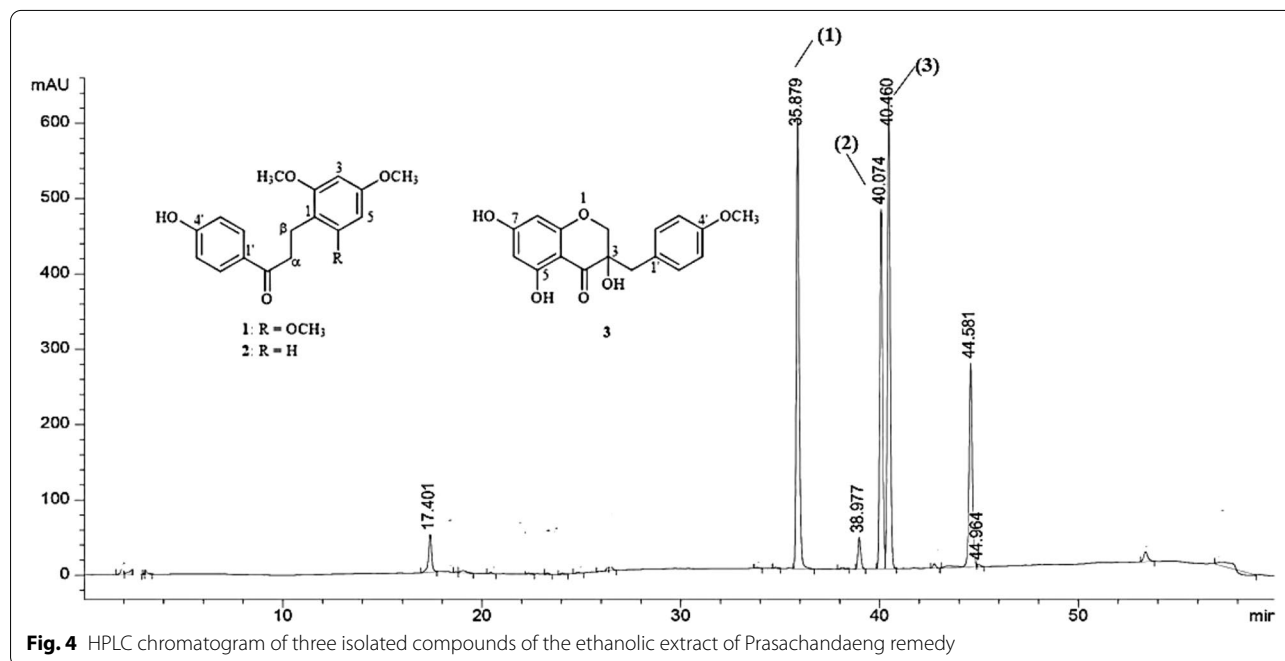


Table 6 IC₅₀ µg/mL ± SEM of cytotoxicity of PSD remedy and its ingredients against two types of cancer cell lines and one non-cancerous cell line using Sulforhodamine B (SRB) assay (n = 3)

Plant species	Code	%Yield (w/w)	IC ₅₀ µg/mL ± SEM and Selective index (SI)		
			HepG2	KKU-M156	HaCaT
<i>Bouea macrophylla</i> Griff	BM95	3.52	36.15 ± 0.54**** (SI = ND)	60.53 ± 1.08**** (SI = ND)	> 100****
<i>Caesalpinia sappan</i> L	CS95	8.17	6.44 ± 0.54**** (SI = 6.7)	4.77 ± 0.57**** (SI = 9.0)	43.14 ± 1.46****
<i>Citrus aurantiifolia</i> (Christm.)	CA95	2.63	92.96 ± 2.22**** (SI = ND)	> 100**** (SI = ND)	> 100****
<i>Dracaena cochinchinensis</i> (Lour.) S.C. Chen	DC95	10.45	7.72 ± 1.87**** (SI = 5.2)	5.27 ± 5.01**** (SI = 7.7)	40.47 ± 0.39****
<i>Heliciopsis terminalis</i> (Kurz)	HT95	1.99	44.50 ± 1.01**** (SI = ND)	43.42 ± 0.29**** (SI = ND)	> 100****
<i>Jasminum sambac</i> (L.) Aiton	JS95	8.59	95.01 ± 0.41**** (SI = ND)	85.77 ± 2.25**** (SI = ND)	> 100****
<i>Kaempferia galanga</i> L	KG95	3.49	7.81 ± 2.39**** (SI = ND)	32.95 ± 3.75**** (SI = ND)	> 100****
<i>Ligusticum chuanxiong</i> Hort	LC95	8.50	11.87 ± 4.43**** (SI = ND)	43.55 ± 4.02**** (SI = ND)	> 100****
<i>Mammea siamensis</i> T. Anderson	MS95	19.41	5.67 ± 0.32**** (SI = 9.0)	7.52 ± 1.05**** (SI = 6.8)	51.20 ± 0.68****
<i>Mesua ferrea</i> L	MF95	11.57	7.10 ± 0.16**** (SI = 7.3)	27.17 ± 2.86**** (SI = 1.9)	51.99 ± 0.37****
<i>Myristica fragrans</i> Houtt	MYF95	2.88	5.67 ± 0.32**** (SI = 4.6)	5.02 ± 3.14**** (SI = 5.2)	26.32 ± 2.67****
<i>Nelumbo nucifera</i> Gaertn	NN95	8.08	> 100**** (SI = ND)	> 100**** (SI = ND)	> 100****
Prasachandaeng remedy	PSD95	13.62	10.45 ± 1.98**** (SI = 5.3)	4.53 ± 0.74**** (SI = 12.2)	55.45 ± 1.73****
Vincristine sulfate	-	-	0.012 ± 0.0005 (SI = 0.00058)	0.0026 ± 0.001 (SI = 0.0026)	0.000007 ± 0.00

ND Not detected, SI Selective index calculated by IC₅₀ of normal cells / IC₅₀ of cancer cells. Data were presented as mean ± SEM and analyzed by one-way ANOVA and Dunnett's multiple comparison tests. Significant different presented the **** p < 0.0001 compared with a standard drug (Vincristine sulfate) in corresponding cell line

HepG2, KKU-M156, and HaCaT. The EtOH extract of PSD remedy (PSD95) exhibited potent cytotoxicity against hepatocellular carcinoma (HepG2) and cholangiocarcinoma (KKU-M156) with IC₅₀ values of 10.45 ± 1.97 (SI = 5.30) and 4.53 ± 0.74 (SI = 12.25) µg/mL, respectively. In addition, PSD95 exhibited moderate cytotoxicity against HaCaT with IC₅₀ values of 55.45 ± 1.73 µg/mL. Some plant ingredients also exhibited strong cytotoxicity against HepG2, i.e., 95% EtOH extract of *C. sappan* (CS95), *D. cochinchinensis* (DC95), *K. galanga* (KG95), *L. chuanxiong* (LC95), *M. siamensis* (MS95), *M. ferrea* (MF95), and *M. fragrans* (MYF95) with IC₅₀ values of 6.44 ± 0.54 (SI = 6.7), 7.72 ± 1.87 (SI = 5.2), 7.81 ± 2.39 (SI = ND), 11.87 ± 4.43 (SI = ND), 5.67 ± 0.32 (SI = 9.0), 7.10 ± 0.16 (SI = 7.3), and 5.67 ± 0.32 (SI = 4.6) µg/mL, respectively. The chemotherapeutic drug (vincristine sulfate) exhibited cytotoxicity against HepG2, KKU-M156, and HaCaT with IC₅₀ values of 0.012 ± 0.0005 (SI = 0.00058), 0.0026 ± 0.001 (SI = 0.0026), and 0.000007 ± 0.00 µg/mL, respectively. The summarized

results of cytotoxic activity are shown in Table 6. The SI index of greater than > 3 shows a good selectivity index of cytotoxic activity using the SRB assay [15, 16]. The selectivity index (SI) in our study is shown in Table 6. The results indicate that all crude extracts did not show cytotoxicity towards the human non-cancerous cell line.

Determination of LPS-induced TNF-α production in RAW 264.7 cells

The DC95 and PSD95 were investigated for anti-inflammatory activity on TNF-α production in the murine macrophage cell line (RAW 264.7). The results are shown in Table 7. The DC95, PSD95 and acetaminophen (ACP) exhibited anti-inflammatory activity by inhibition of TNF-α production with the percentage values of 71.133 ± 2.806, 45.083 ± 1.814, and 18.657 ± 1.925%, respectively. The DC95 and PSD95 showed significant difference from acetaminophen (p > 0.05) in their anti-TNF-α production. The results also showed that DC95 and PSD95 exhibited

Table 7 Anti-inflammatory on LPS-induced tumor necrosis factor- α production of *D.cochinchinensis* and PSD remedy

Plant species	CODE	Inhibition of TNF- α production	
		%Inhibition and [%cytotoxicity] at conc. 100 μ g/mL	IC ₅₀ μ g/mL
<i>D.cochinchinensis</i>	DC95	71.133 \pm 2.806 ^{****} [25.676 \pm 3.451 [†]]	82.070 \pm 7.570 [*]
Prasachandaeng remedy	PSD95	45.083 \pm 1.814 ^{***} [18.986 \pm 0.460 [†]]	> 100
Acetaminophen	ACP	18.657 \pm 1.925 [59.222 \pm 7.693]	> 100

(-) Indicates not tested. Data were analyzed by one-way ANOVA and Dunnett's multiple comparison tests. Significant difference (†) is when * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$ in comparison with an acetaminophen (ACP) ($n = 3$)

inhibitory activity on TNF- α production 4, and 2.5-fold, respectively, higher than the standard anti-pyretic / analgesic drug, acetaminophen.

Discussion

In this study, the ethnopharmacological wisdom of TTM for treating toxic (chronic) fever starting with the need to reduce high temperature (Pit—Ta; fire element) provided initial guidance). According to the TTM, PSD remedy has bitter and cold flavors that can reduce toxic fever. Furthermore, the ingredients exhibited several flavors, i.e., astringent, fragrant, spicy. These combinations of ingredients and the amelioration of related symptoms of chronic diseases are linked. The flavors of PSD ingredients and their bioactivity are shown in Table 8. The 95% ethanolic extract of PSD remedy extract (PSD95) exhibited strong cytotoxic activity against two types of human cancer cell lines, i.e., hepatocellular carcinoma cell line (HepG2) and cholangiocarcinoma cell line (KKU-M156). Interestingly, its ingredients of PSD remedy, i.e., *C.sappan* (CS95), *D.cochinchinensis* (DC95), *M.siamensis* (MF95), and *M. fragrans* (MYF95) also exhibited strong cytotoxic activity against cholangiocarcinoma cell line (KKU-M156) with IC₅₀ values less than 10 μ g/mL, respectively. The previous study demonstrated that the 70% ethanolic extract of *C.sappan* showed cytotoxic activity against hepatocellular carcinoma (HepG2) cell line [24]. Additionally, the *Streptomyces* sp. HUST012 (SPE-B5.4) was isolated from the heartwood of *D.cochinchinensis* resulted in potent cytotoxic activity against hepatocellular carcinoma cell line (HepG2) with an IC₅₀ value of 0.23 μ g/mL [25]. The results of this study were in accordance with previous study that demonstrated the *C.sappan* and *D.cochinchinensis* exhibited cytotoxic activity against hepatocellular carcinoma (HepG2) with the IC₅₀ values less than 20 μ g/mL. On the other hand, the extract of *M.ferrea* showed comparably modest cytotoxic activity using MTT assay against

cholangiocarcinoma cell line (CL-60) with IC₅₀ value of 48.23 μ g/mL [26].

Compound 1 exhibited cytotoxicity against HepG2 and KKU-M146 with the IC₅₀ values of 20.02 \pm 0.46 and 21.26 \pm 3.17 μ g/mL, respectively (Table 3). Current evidence indicates that retrodihydrochalcones can exert antiproliferation activity against human cancer cell lines when they carry hydroxy substituents in appropriate positions. The active compounds share two para-hydroxybenzene rings connected by a chain of three carbon atoms. This is in sharp contrast to isoflavones which are regarded as analogs of dihydroxystilbene in which two para-hydroxybenzene rings are connected via a chain of two carbon atoms. These findings regarding the structure–activity relationship and antiproliferation activity require further investigation [21]. Our current findings demonstrate cytotoxicity of 4-Hydroxy-2,4'-dimethoxydihydrochalcone (2) against HepG2 and KKU-M146 with the IC₅₀ values of 20.71 \pm 0.49 and 33.21 \pm 2.10 μ g/mL, respectively. There has been no previous report on cytotoxic activity of 4-Hydroxy-2,4'-dimethoxydihydrochalcone (2) against cancer cell lines. This is also the first scientific evidence of its cytotoxic activity against cancer cell lines in comparison with a non-cancerous cell line.

Eucomol (3) has three OH groups that can increase the bioactivity [23]. This is the first report of isolation of Eucomol (3) from the heartwood of an ethanolic extract of *D. cochinchinensis*. In our investigations, we have discovered flavonoids that are an important class of natural products. They belong to a class of plant secondary metabolites having a polyphenolic structure widely found in fruits, vegetables, and herbs. There are several well characterized bioactivities of flavonoids such as antioxidant, anti-inflammatory, and anti-carcinogenic properties [47]. The results of this study are in accordance with a previous study which demonstrated that *D. cochinchinensis* exhibited cytotoxic activity against hepatocellular carcinoma (HepG2) with IC₅₀ values less than < 20 μ g/mL

Table 8 The flavors of herbal ingredients and relationship between the usage of Thai traditional medicine (TTM) and evidence-based approach of herbal medicine of PSD remedy and its ingredients

Plant species (Family)	Thai name	Flavor	TTM used ^{a,b}	Evidence-based approach of herbal medicine
<i>Bouea macrophylla</i> Griff. (Anacardiaceae)	Ma-Prang	Flavorless	Antipyretic ^a , diuretic ^b	Antioxidant [27]
<i>Caesalpinia sappan</i> L. (Leguminosae)	Faang	Astringent	Anti-inflammatory ^a , wound healing ^a , detoxification ^a , increase blood circulation ^a , blood tonic ^a , cardiotoxic ^a , chicken pox ^b	Anti-inflammatory, Antioxidant, Cytotoxic [24, 28, 29]
<i>Citrus aurantiifolia</i> (Christm.) Swingle (Rutaceae)	Ma-Nao	Flavorless	Antipyretic ^a , detoxification ^a , increase blood circulation ^a , common cold influenza ^a , diuretic ^b	Cytotoxic, Anti-inflammatory [30–32]
<i>Dracaena cochinchinensis</i> (Lour.) S.C. Chen (Dracaenaceae)	Chan-Daeng	Bitter&cold	Antipyretic ^a , anti-inflammatory ^a , wound healing ^a , detoxification ^a , increase blood circulation ^a , blood tonic ^a	Anti-inflammatory, Cytotoxic, Antipyretic [25, 33, 34]
<i>Heliciopsis terminalis</i> (Kurz) Sleumer (Proteaceae)	Mhuad-Kon	Flavorless	Common cold influenza ^a , diuretic ^b	Anti-inflammatory [10]
<i>Jasminum sambac</i> (L.) Aiton (Oleaceae)	Ma-Li	Fragrant	Antipyretic ^a , cardiotoxic ^a , sleep disorder ^b , headache ^b	Anti-inflammatory, and antipyretic [35]
<i>Kaempferia galanga</i> L. (Zingiberaceae)	Por-Hom	Spicy	Common cold influenza ^a , reducing the dyspepsia ^a , increase blood circulation ^b	Anti-inflammatory [36]
<i>Ligusticum chuanxiong</i> Hort. (Umbelliferae)	Khod-Hua-Bua	Spicy	Anti-inflammatory ^a , wound healing ^a , increase blood circulation ^b	Cytotoxic [37]
<i>Mammea siamensis</i> T. Anderson. (Calophyllaceae)	Sa-Ra-Pee	Fragrant	Antipyretic ^a , cardiotoxic ^a	Cytotoxic, Anti-inflammatory [38, 39]
<i>Mesua ferrea</i> L. (Calophyllaceae)	Boon-Nak	Fragrant	Blood tonic ^a , antipyretic ^a headache ^a , cardiotoxic ^a	Cytotoxic [26, 40]
<i>Myristica fragrans</i> Houtt. (Myristicaceae)	Chan-Thet	Fragrant	Enhancing immune function ^a , blood tonic ^a , cardiotoxic ^a , antipyretic ^a , headache ^a , dyspepsia ^a	Cytotoxic, Anti-inflammatory [41–43]
<i>Nelumbo nucifera</i> Gaertn. (Nelumbonaceae)	Bua-Luang	Fragrant	Cardiotoxic ^a , antipyretic ^a	Cytotoxic, Antipyretic [44, 45]
Prasachandaeng remedy	-	Bitter&cold	Reduction of fever (chronic fever), and treatment of aphthous stomatitis	Anti-inflammatory, antipyretic [10, 46]

(-) Indicates the data not shown. ^aThai traditional medical textbook, The Rehabilitation Foundation for Thai Traditional Medicine and Ayurved Thamrong School, 2007. ^bThe usages according to Thai traditional doctor

[25]. This is the first report of PSD95 and DC95 on anti-TNF- α production in RAW264.7. Both crude extracts showed the % inhibition of anti-inflammatory activity via TNF- α production higher than the positive control acetaminophen. Therefore, these results support the use of PSD95 and DC95 for treating chronic fever based on their ability to inhibit the pro-inflammatory cytokine-related carcinogen than a well established drug used clinically. However, we investigated the pharmacology of PSD remedy in comparison with a drug known to possess antipyretic activity in animal models [46]. Quality control of the chemical contents of PSD95 with a validated HPLC method was determined. The study provided preliminary data on the major chemical constituents of the PSD remedy. However, further studies on molecular docking of the pure compounds and additional biological and pharmacological characterization are warranted.

Conclusion

The scientific evidence detailed in these investigations suggests that the three isolated compounds discovered had anti-cancer proliferative activity. The PSD remedy exhibited potent cytotoxic activity against hepatocellular carcinoma (HepG2) and cholangiocarcinoma (KKU-M156). In fact, PSD remedy exhibited a greater anti-inflammatory activity as measured by inhibition of TNF- α production than acetaminophen. The results of this study support the Thai traditional wisdom that the uses the herbal combination as ingredients in traditional remedies may be effective as medicines for cancer, at least in part through their anti-inflammatory, and antipyretic activities. As a result of this study, the Thai traditional practitioners and folk doctors that use PSD remedy for toxic fever in liver and bile duct cancer patients also

have additional scientific evidence underlying the rationale of their continued clinical use.

Abbreviations

ATCC: American type culture collection; CHCl₃: Chloroform; CC: Column chromatography; DMEM: Dulbecco's modified eagle medium; DMSO: Dimethylsulfoxide;; ELISA kit: Enzyme-linked immunosorbent assay; EtOAc: Ethyl acetate; EtOH: Ethanol; FBS: Fetal bovine serum; HaCaT: Human keratinocyte cell line; HCl: Hydrochloric acid; HepG2: Hepatocarcinoma cell line; HPLC: High performance liquid chromatography; KLU-M156: Cholangiocarcinoma cell line; LPS: Lipopolysaccharide; MEM: Minimum essential media; MeOH: Methanol; NMR: Nuclear magnetic resonance; PBS: Phosphate buffered saline; PSD: Prasachandaeng; SEM: Standard error of means; SI: Selectivity index; SRB assay: Sulforhodamine B assay; TCA: Trichloroacetic acid; TNF- α : Tumor necrosis factor- α ; TLC: Thin layer chromatography; TTM: Thai traditional medicine; VLC: Vacuum liquid chromatography.

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Authors' contributions

Nuntika Prommee (NP) is PhD candidate under supervision of Arunporn Itharat (AI), Pakakrong Thongdeeying (PK) assisted in identification and elucidation of isolated compounds; Sunita Makchuchit (SM) taught and conducted bioassays, Weerachai Pipatratanaseree (WP) helped in isolation of compounds and their analysis, Adis Tasanarong (AT) help with discussion of the results, Arunporn Itharat (AI) is project manager and NP's supervisor and responsible for methodology, investigation, writing original draft. Buncha Oorakul (BO): reviewing and editing. Neal M. Davies (ND): interpretation of results, reviewing and editing and language assistance, and supervision. The author(s) read and approved the final manuscript.

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Availability of data and materials

Dataset of this manuscript has not been deposited in any reposition. All datasets and materials are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The biological activities of this study were approved by the Biosafety Committee of Thammasat University, Thailand. They approved this experiment under Biosafety Level 1 (BSL I, Number 092/2018).

Consent for publication

Not applicable.

Competing interests

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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